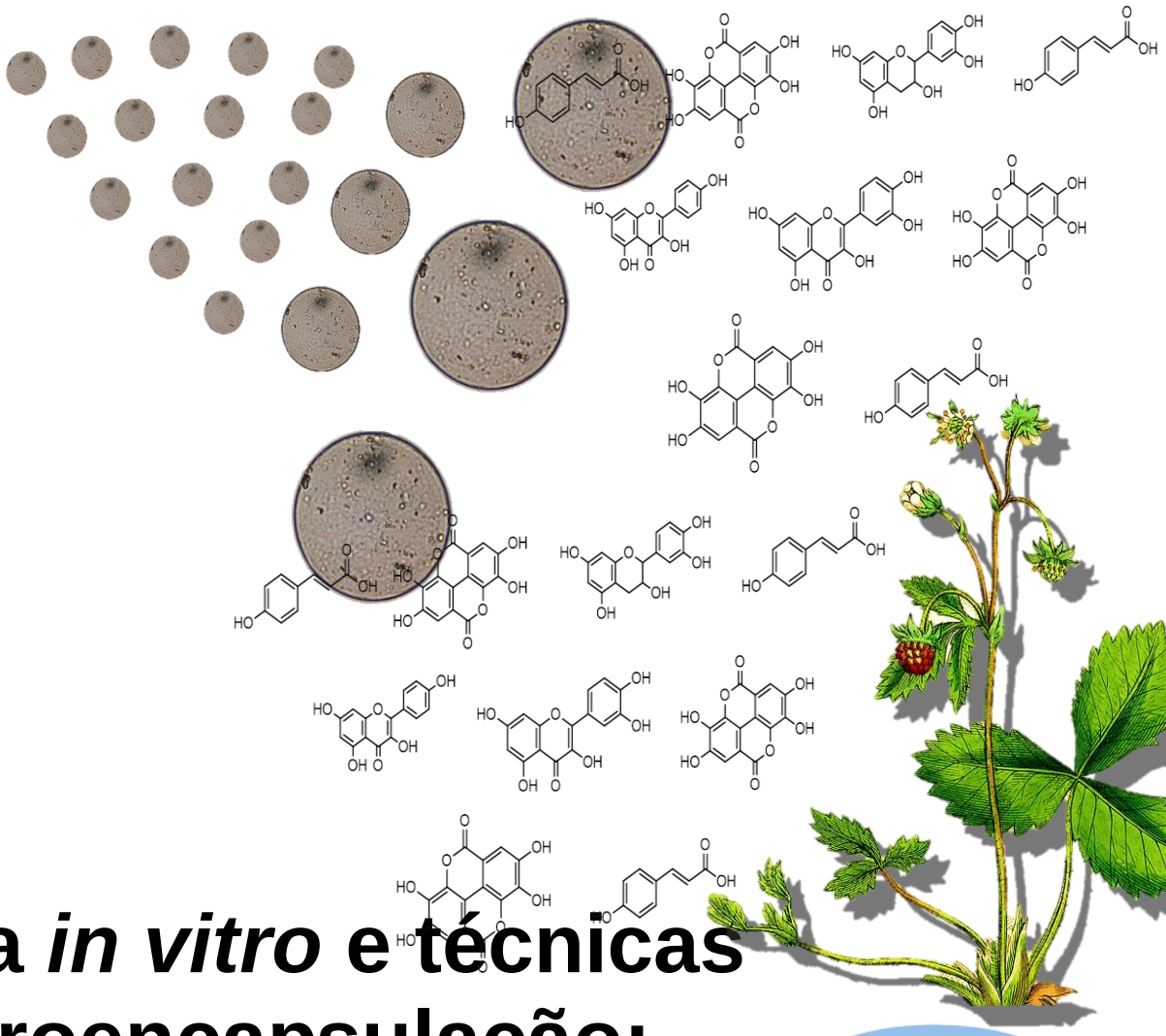

Cultura *in vitro* e técnicas de microencapsulação: aumento da produção e da estabilidade de compostos bioativos de espécies vegetais

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"*Para termos uma noção do pouco que valemos, basta subtrair ao que somos o que aprendemos, o que lemos, o que vivemos com os outros. É só ver o que fica. Coisa pouca. Sozinho quase ninguém é quase nada. É somente juntos que podemos ser alguma coisa*."

Miguel Esteves Cardoso

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“eles não sabem que o sonho
é vinho, é espuma, é fermento,
bichinho álacre e sedento,
de focinho pontiagudo,
que fossa através de tudo
num perpétuo movimento”

em Movimento Perpétuo, “Pedra Filosofal” (1956) de António Gedeão

Para os meus avós

Resumo

A procura de novos produtos alimentares relaciona-se com a necessidade dos consumidores adotarem um estilo de vida saudável para que a médio-longo prazo não se assista a um aumento exponencial da incidência de doenças crónicas. Os novos produtos apresentam, muitas vezes, propriedades funcionais benéficas para a saúde do consumidor, para além das suas caraterísticas nutricionais intrínsecas. Estes efeitos benéficos são conseguidos pela presença ou enriquecimento com compostos/extratos bioativos provenientes de matrizes naturais, nomeadamente vegetais.

A imensa procura destes bioativos naturais suscita a necessidade de assegurar a manutenção das populações de plantas e preservação do seu *habitat*, evitando a perda de diversidade genética, pelo que é crucial a utilização de novas técnicas de produção e obtenção de bioativos. A cultura *in vitro*, através de técnicas de micropropagação e elicitação de vias metabólicas, surge como uma alternativa viável e sustentável para a produção desses bioativos com aplicabilidade alimentar.

No entanto, a perecibilidade de alguns bioativos durante o processamento e armazenamento, associada à sua degradação após ingestão é outra preocupação que surge associada a esta tendência de mercado, uma vez que condiciona a sua utilização em produtos alimentares e posterior eficácia após consumo. A microencapsulação dos bioativos procura dar resposta a esta preocupação, permitindo a sua retenção dentro de uma cápsula que assegura estabilidade e que irá libertar o seu conteúdo num determinado alvo de forma a aumentar a sua eficácia.

A presente dissertação envolve estudos de aplicação das duas ferramentas biotecnológicas (cultura *in vitro* e microencapsulação) na área dos bioativos de origem vegetal, tendo como objetivos respetivos a obtenção de uma maior quantidade de bioativos, nomeadamente compostos fenólicos, e a proteção/estabilização desses compostos para posterior aplicação numa matriz alimentar.

Numa primeira fase, foi realizado um *screening* a várias espécies de plantas tradicionalmente consumidas no Nordeste Transmontano, e ainda pouco estudadas, de forma a encontrar a mais promissora no que respeita ao teor em compostos bioativos, para posterior aplicação das técnicas de cultura *in vitro* e de microencapsulação. Testaram-se amostras comerciais e silvestres de *Achillea millefolium* L. (partes aéreas de mil-folhas), *Laurus nobilis* L. (folhas de loureiro) e *Fragaria vesca* L. (raízes, partes vegetativas e fruto de morangueiro) e amostras silvestres de *Taraxacum* sect. Ruderalia (partes vegetativas e flores de dente-de-leão); quer na forma desidratada quer em extratos hidrometanólicos e aquosos (obtidos por infusão e decocção).

A caraterização nutricional das amostras envolveu a determinação de gordura, proteínas, cinzas, hidratos de carbono e fibras por métodos oficiais de análise de alimentos. Foram também analisados os perfis em ácidos gordos (cromatografia gasosa acoplada a um detetor de ionização de chama), açúcares (cromatografia líquida de alta eficiência- HPLC- acoplada a um detetor de índice de refração), ácidos orgânicos (HPLC acoplada a um detetor de fotodíodos- PDA), tocoferóis e folatos (HPLC acoplada a um detetor de fluorescência) e minerais (espectroscopia de absorção atómica). Os compostos fenólicos foram analisados por HPLC-PDA e ionização por dispersão de eletrões acoplada a um detetor de espetrometria de massa.

Os extratos hidrometanólicos e aquosos foram estudados relativamente ao seu potencial antioxidante, tendo sido aplicados quatro métodos distintos: atividade captadora de radicais 2,2-difenil-1-picril-hidrazilo, poder redutor, inibição da descoloração do β-caroteno e inibição da peroxidação lipídica através do ensaio das espécies reativas do ácido tiobarbitúrico- TBARS. As propriedades citotóxicas dos extratos foram também estudadas em linhas celulares tumorais humanas (MCF-7- carcinoma de mama, NCI-H460- carcinoma de pulmão, HCT 15- carcinoma de cólon, HeLa- carcinoma cervical e HepG2- carcinoma hepatocelular) e em culturas primárias de células de fígado de porco (PLP2), através do ensaio da sulforrodamina B. As propriedades antimicrobianas foram testadas usando estirpes de coleção e bactérias isoladas clinicamente, através da técnica de microdiluição acoplada ao método colorimétrico de deteção rápida com cloreto de *p*-iodonitrotetrazólio- INT); e pela inibição da produção de biofilme em estirpes de bactérias isoladas clinicamente. Os resultados obtidos mostraram que todas as amostras estudadas são potenciais fontes de compostos com elevado valor nutricional e bioativo, nomeadamente pelas suas propriedades citotóxicas (mil-folhas e loureiro) e antimicrobianas (loureiro e morangueiro). As amostras silvestres revelaram, em geral, um maior potencial comparativamente às comerciais; e os extratos aquosos, na grande maioria das amostras, mostraram maior potencial antioxidante. No entanto, foi com a amostra de morangueiro silvestre que se obtiveram os melhores resultados, mostrando-se esta espécie a mais promissora para estabelecer em cultura *in vitro* e obter bioativos para posterior microencapsulação.

Assim, procedeu-se à esterilização do explante (fruto de morangueiro), à germinação das sementes e à aplicação de diferentes concentrações dos fitorreguladores IBA (ácido indolbutírico) e BAP (benzilaminopurina). Da massa vegetal obtida após crescimento controlado, fizeram-se novamente os ensaios de caraterização química e avaliação de propriedades bioativas em extratos hidrometanólicos e aquosos (obtidos por infusão e decocção). As partes vegetativas cultivadas *in vitro* revelaram maior quantidade de proteínas, ácidos gordos polinsaturados, açúcares e ácidos orgânicos

comparativamente ao seu homólogo silvestre; os extratos hidrometanólicos revelaram também um maior atividade antioxidante do que os previamente obtidos. Em termos de compostos fenólicos, foram obtidos compostos diferentes mas com elevada correlação com a sua bioatividade.

Finalmente, o extrato mais bioativo (infusão das partes vegetativas) foi estabilizado por microencapsulação (técnica de atomização/coagulação), tendo-se procedido à caraterização das microesferas por microscopia ótica, microscopia eletrónica de varrimento espetroscopia de infravermelho, bem como à avaliação da eficiência de encapsulação por análise do composto maioritário por HPLC. O extrato foi posteriormente incorporado, na forma livre e microencapsulada, em gelatina de *k*-carragenina. A técnica de encapsulação provou ser eficaz tendo-se obtido uma eficiência de encapsulação de aproximadamente 95%. A integridade das microesferas enriquecidas assim como a sua capacidade de reidratação não foi alterada após a preparação da gelatina a altas temperaturas (100 °C). A gelatina com o extrato livre mostrou menor atividade antioxidante evidenciando uma degradação do extrato aquando da preparação da gelatina; pelo contrário, a gelatina com o extrato microencapsulado não revelou qualquer bioatividade o que comprova que o extrato ficou retido e protegido dentro da microesfera até ao seu posterior consumo.

Com o presente estudo demonstrou-se que a técnica de cultura de células e tecidos vegetais é viável para a produção de compostos bioativos e que a sua encapsulação para utilização em matrizes alimentares representa uma grande melhoria para a indústria alimentar uma vez que o consumidor pode beneficiar de todo o potencial bioativo pretendido.

Palavras-chave: Plantas aromáticas e medicinais, cultura in vitro*, microencapsulação, nutracêuticos, compostos bioativos*

Abstract

The demand for new food products relates to the need for consumers to adopt a healthy lifestyle so that in the medium to long term there is no exponential increase in the incidence of chronic diseases. The new products often have functional properties beneficial to the health of the consumer, in addition to their intrinsic nutritional characteristics. These beneficial effects are achieved by the presence or enrichment with bioactive compounds/extracts from natural matrices, namely plants.

The immense demand for these natural bioactives raises the need to ensure the maintenance of plant populations and the preservation of their habitat, avoiding the loss of genetic diversity, so it is crucial the use of new bioactive production and obtainment techniques. *In vitro* culture, through micropropagation techniques and elicitation of metabolic pathways, appears as a viable and sustainable alternative for the production of these bioactives with food applicability.

However, the pereceability of some bioactives during processing and storage associated with their degradation after ingestion is another concern that is associated with this market trend, since it conditions their use in food products and subsequent efficacy after consumption. The microencapsulation of bioactives seeks to respond to this concern by allowing their retention within a capsule which ensures stability and which will release its contents at a given target in order to increase its effectiveness.

The present dissertation involves the study of the two biotechnological tools (*in vitro* culture and microencapsulation) in the area of plant bioactive, with the respective objectives to obtain a greater amount of bioactives, namely phenolic compounds, and the protection/stabilization of these compounds, for later application in a food matrix.

In a first phase, a screening was done on several species of plants traditionally consumed in the Portuguese Northeast region, and still scarcely studied, in order to find the most promising ones regarding the content of bioactive compounds, for later application of *in vitro* culture techniques and microencapsulation. Commercial and wild samples of *Achillea millefolium* L. (aerial parts of yarrow leaves), *Laurus nobilis* L. (laurel leaves) and *Fragaria vesca* L. (roots, vegetative parts and strawberry fruit) and wild samples of *Taraxacum* Sect. Ruderalia (vegetative parts and flowers of dandelion); either in the dehydrated form or in hydromethanolic and aqueous extracts (obtained by infusion and decoction).

The nutritional characterization of the samples involved the determination of fat, proteins, ash, carbohydrates and fibers by official methods of food analysis. Were also analyzed the fatty acid profiles (gas chromatography coupled to a flame ionization detector), sugars (HPLC-coupled to a refraction index detector), organic acids (HPLC coupled to a

detector of photodiodes-PDA), tocopherols and folates (HPLC coupled to a fluorescence detector) and minerals (atomic absorption spectroscopy). The phenolic compounds were analyzed by HPLC-PDA and electron dispersion ionization coupled to a mass spectrometry detector.

The hydromethanolic and aqueous extracts were studied for their antioxidant potential and four different methods were applied: 2,2-diphenyl-1-picryl-hydrazyl radical scavenging activity, reducing power, β-carotene bleaching inhibition and inhibition of lipid peroxidation by reactive species of thiobarbituric acid-TBARS. The cytotoxic properties of the extracts were also studied in human tumor cell lines (MCF-7- breast carcinoma, NCI-H460- lung carcinoma, HCT-15- colon carcinoma, HeLa- cervix carcinoma and HepG2-hepatocellular carcinoma) (PLP2) by the sulforhodamine B assay. The antimicrobial properties were tested using collection strains and clinically isolated bacteria by the microdilution technique coupled with the rapid detection colorimetric method with *p*-iodonitrotetrazolium chloride - INT); and inhibition of biofilm production in strains of clinically isolated bacteria. The results showed that all the studied samples are potential sources of compounds with high nutritional and bioactive value, mainly due to its cytotoxic properties (yarrow leaves and laurel) and antimicrobial (laurel and strawberry). Wild samples showed, in general, greater potential compared to commercial ones; and the aqueous extracts, in the majority of the samples, showed greater antioxidant potential. However, it was with the wild strawberry sample that the best results were obtained, showing the most promising species to establish *in vitro* culture and to obtain bioactives for later microencapsulation.

Thus, preceded to the explant (strawberry fruit) sterilization, germination of the seeds and application of different concentrations of the growth regulators IBA (indolbutyric acid) and BAP (benzilaminopurine). The chemical characterization and evaluation of bioactive properties in hydromethanolic and aqueous extracts (obtained by infusion and decoction) were performed again after the controlled growth. Vegetative parts grown *in vitro* revealed higher amounts of proteins, polyunsaturated fatty acids, sugars and organic acids compared to their wild counterpart; the hydromethanolic extracts also showed a higher antioxidant activity than the previously obtained ones. In terms of phenolic compounds, different compounds were obtained but with high correlation with their bioactivity.

Finally, the most bioactive extract (infusion of the vegetative parts) was stabilized by microencapsulation (atomization/coagulation technique). The microspheres were characterized by optical microscopy, scanning electron microscopy and infrared spectroscopy, as well as the evaluation of encapsulation efficiency by HPLC analysis of the major compound. The extract was further incorporated, in free and microencapsulated form, into *k*-carrageenan gelatin. The encapsulation technique proved to be effective having achieved an encapsulation efficiency of approximately 95%. The integrity of the enriched

microspheres as well as their rehydration capacity was not altered after gelatin preparation at high temperatures (100 °C). The gelatin with the free extract showed lower antioxidant activity evidencing a degradation of the extract when preparing the gelatin; On the contrary, the gelatin with the microencapsulated extract did not show any bioactivity which proves that the extract was retained and protected inside the microsphere until its later consumption.

With the present study it was demonstrated that the technique of plant cell and tissue culture is viable for the production of bioactive compounds and its encapsulation for use in food matrices represents a great improvement for the food industry since the consumer can benefit of the intended bioactive potential.

Keywords: Aromatic and medicinal plants; in vitro *culture; microencapsulation; nutraceuticals; bioactive compounds*

Lista de publicações

Capítulos de livro:

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2. Maria Inês Dias, Lillian Barros, M. Beatriz P.P. Oliveira, Celestino Santos-Buelga, Maria Filomena Barreiro, Isabel C.F.R. Ferreira « Nova formulação nutracêutica à base de extratos fenólicos microencapsulados de partes vegetativas de *Fragaria vesca* L. silvestre» Publicado XIII Encontro de Química dos Alimentos, Porto Portugal.

Comunicações orais

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Lista de abreviaturas e símbolos

Visto tratar-se de um documento bilingue, a explicação da abreviatura/símbolo aparece na língua correspondente ao texto em que aparece mencionada.

2,4-D	Ácido 2,4-diclorofenoxiacético
AAS	Atomic absorption spectroscopy
ABTS	Ácido 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfónico)
ANA	Ácido naftalenoacético
ANOVA	Análise de variância
AOAC	Associação Oficial de Químicos Analíticos/Association of Oficial Analytical Chemists
ATCC	Coleção de culturas tipo Americana/American type culture collection
BAP	Benzilaminopurina/Benzylaminopurine
CD	Circular dichroism
CFU	Colony-forming unit
DAD	Diode array detetor
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
DPPH	2,2-difenil-1-picril-hidrazilo/2,2-Diphenyl-1-picrylhydrazyl
DR	Dry residue
dw	Dry weight
EC_{50}	Effective concentration achieving 50% of antioxidant activity or 0.5 absorbance in reducing power assay
EDTA	Ethylenediaminetetraacetic acid
EE	Encapsulation efficiency
EFSA	Autoridade Europeia para a Segurança Alimentar/European Food Safety Authority
EMM	Estimated marginal means
ESBL	Extended spectrum betalactamase
ESI	Electrospray ionization
EUA	Estados Unidos da América
EUCAST	European committee on antimicrobial susceptibility testing
ex.	Exemplo
FAME	Fatty acids methyl ester

FAO	Organização das Nações Unidas para a alimentação e a agricultura/Food and Agricultural Organization
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FID	Flame ionization detetor
FL	Fluorescence
FTIR	Fourier transform infrared spectroscopy
fw	Fresh weight
GC	Gas-chromatography
GI_{50}	Sample concentration that inhibited 50% of the net cell growth
GLM	General linear model
GRAS	Geralmente reconhecidos como seguros/Generally recognized as safe
HBSS	Hank's balanced salt solution
HHDP	Hexahydroxydiphenic acid
HPLC	Cromatografia líquida de alta eficiência/High-performance liquid chromatography
HRF	Heterocyclic ring fissions
IAA	Ácido 3-indolacético
IAEA	Divisão de técnicas nucleares para a alimentação e agricultura
IBA	Ácido índolbutírico/Indolebutyric acid
INT	Cloreto de *p*-iodonitrotetrazólio/*p*-Iodonitrotetrazolium chloride
IS	Internal standart
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass-to-charge ratio
MA	Malt agar
MBC	Minimum bactericidal concentration
MDA-TBA	Malondialdehyde-thiobarbituric acid
M_{e-ne}	Non-encapsulated extract remaining after the encapsulation process
M_{e-t}	Theoretical amount of extract, i.e. the amount of extract used in the microencapsulation process
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectometry
MS^2	Second stage of mass spectrometry

mu	Mass unit
MUFA	Monounsaturated fatty acids
n6/n3	Omega-6 to omega-3 ration
na	Not applicable
nd	Not detected
NMR	Nuclear magnetic resonance
NRV	Nutritional references values
ODassay	Optical density of the assay
ODcontrol	Optical density of the control
OM	Optical microscopy
ORAC	Oxygen radical absorbance capacity
PAC	Proanthocyanidins
PAL	Fenilalanina amónia-liase
PCA	Principal component analysis
PCL	Policaprolactona
PDA	Photodiode array detector
PEG	Polietileno glicol
PGPR	Poliglicerol poliricinoleato
PLA	Poli-D, L-láctido
PUFA	Polyunsaturated fatty acids
R	Resistant
R^2	Coefficient of determination
RDA	Reference daily intake
RDA	Retro-Diels-Alder
RDA	Recommended dietary allowance
RI	Refraction index
Rt	Retention time
S	Susceptible
SD	Standard deviation
SEM	Scanning electron microscope
SFA	Saturated fatty acids
SPSS	Statistical package for the social sciences
SRB	Sulphorhodamine B
TA	Total anthocyanins
TBARS	Espécies reativas do ácido tiobarbitúrico/Thiobarbituric acid reactive substances

TCA	Trichloroacetic acid
TDF	Total dihydroflavonols
TdhF	Total dihydroflavonols
TEAC	Trolox equivalent antioxidant capacity
TED	Total ellagic acid derivatives
TF	Total flavonoids
TF3O	Total flavan-3-ols
TPA	Total phenolic acids
tr	Traces
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TSB	Tryptic soy broth
UE	União europeia
UFLC	Ultra-fast liquid chromatography
UV	Radiação ultravioleta
v/v	Volumetric percentage
w/w	Percentage solution
WHO	Organização mundial de saúde/World health organization
λ_{max}	Wavelength of maximum absorption

1.

Motivação, objetivos e estrutura da tese

No presente capítulo identificam-se as principais motivações para o desenvolvimento desta tese, apresentando a problemática da produção e utilização de compostos bioativos. Enumeram-se também os principais objetivos, apresentam-se as tarefas para os atingir, bem como os artigos científicos resultantes do trabalho desenvolvido nesta tese.

1.1. Motivação da Tese

As estatísticas apontam para que haja um aumento exponencial na incidência de doenças crónicas, como o cancro, doenças cardiovasculares e respiratórias, diabetes, entre outras (WHO, 2005). Sabe-se também que muitas destas doenças estão diretamente relacionadas com o estilo de vida adotado mundialmente no último século, incluindo o sedentarismo e a má alimentação.

Por este facto, tem-se assistido a uma crescente procura por parte dos consumidores por novos produtos alimentares que complementem características nutricionais com propriedades funcionais, proporcionando uma fonte adicional de compostos benéficos para a saúde. Estes novos produtos alimentares podem ser conseguidos pela introdução de ingredientes naturais provenientes de matrizes tais como, plantas aromáticas e medicinais. Estas têm vindo a ser usadas desde tempos ancestrais dado as suas características organoléticas, terapêuticas e medicinais, representando por isso, ainda hoje, um marco para a etnomedicina na procura de novos compostos bioativos (Fabricant & Farnsworth, 2001).

Dado o aumento da procura por estes novos produtos à base de ingredientes naturais, é necessário encontrar uma resposta eficaz para a sua produção, nomeadamente sem conduzir a perdas de populações de plantas, degradação dos habitats ou perda da diversidade genética que, em último caso, pode levar à extinção de espécies (Schippmann et al, 2002; Roberto et al., 201). Surge assim a cultura de células e tecidos vegetais como uma alternativa sustentável e viável que responde a esta problemática estimulando a produção por técnicas de micropropagação e elicitação. Esta técnica está endossada pela FAO (Organização das Nações Unidas para a Alimentação e a Agricultura) como permitida para a produção de compostos bioativos com aplicabilidade na indústria alimentar. Adicionalmente, existem já uma série de diretrizes publicadas pela FAO em conjunto com a IAEA (Divisão de técnicas nucleares para a alimentação e agricultura), visando a sua implementação de forma sustentável e economicamente viável (FAO/IAEA, 2002).

Outra problemática relacionada com os compostos bioativos é a sua perecibilidade; estes podem apresentar tendência para a degradação quando processados, durante o armazenamento ou até mesmo após ingestão, o que condiciona a sua utilização direta nos alimentos limitando o desenvolvimento de novos alimentos funcionais na indústria alimentar (Espín et al., 2007; Joye et al., 2014).

A técnica proposta para colmatar esta fragilidade dos compostos bioativos é a microencapsulação, técnica que tem vindo a ser usada, já há já algumas décadas, em outros setores industriais, nomeadamente têxtil, agrícola e farmacêutico (Martins et al., 2014). No que respeita a indústria alimentar, a microencapsulação tem vindo a despertar um interesse crescente pois permite não só a proteção eficaz dos compostos bioativos, como

também assegura a sua estabilidade e permite uma libertação controlada e/ou localizada no organismo, aumentando assim a eficácia destes novos ingredientes naturais (Kuang et al., 2010; Nazzaro et al., 2012).

Neste contexto, o grande objecto de estudo desta tese são os compostos bioativos, mais propriamente os compostos fenólicos. Estes compostos são reconhecidos pelas suas propriedades biológicas, mas também bioativas, apresentando propriedades anticancerígenas e antifibrilogénicas (Quideau et al., 2011; Carocho & Ferreira, 2013). De realçar que o seu consumo diário resulta em efeitos benéficos para a saúde do consumidor a longo prazo e, por isso, tem motivado muitos estudos relacionados com a sua utilização na alimentação. Assim, torna-se importante a sua obtenção em quantidade através de técnicas de cultura de células e tecidos, mas também a sua protecção e aplicação em matrizes alimentares através de técnicas de microencapsulação.

Assim, no presente trabalho, aplicaram-se duas ferramentas à área dos bioativos naturais: (i) a técnica de cultura *in vitro* que visou estudar a intensificação da produção de compostos bioativos, mais especificamente compostos fenólicos; (ii) a técnica de microencapsulação como via de viabilização do uso destes ingredientes funcionais em matrizes alimentares, sem perda da sua bioatividade.

1.2. Objetivos

O objectivo principal deste trabalho consistiu na aplicação de duas tecnologias à obtenção de compostos bioativos de espécies vegetais, uma destinada à produção em larga escala destes fitoquímicos, nomeadamente compostos fenólicos (cultura *in vitro*), e outra visando colmatar a fragilidade que estes apresentam na sua forma livre (microencapsulação).

Numa primeira abordagem, selecionaram-se quatro espécies de plantas: *Achillea millefolium* L., *Fragaria vesca* L. *Laurus nobilis* L. e *Taraxacum* sect *Ruderalia* (**Figura 1**), que foram submetidas a estudos de caracterização nutricional e química, bem como à avaliação das propriedades bioativas. Foram determinados os teores de cinzas, proteínas, gordura, minerais (micro e macroelementos), fibra e valor energético, utilizando procedimentos AOAC (Associação Oficial de Químicos Analíticos), bem como a composição individual em ácidos gordos, tocoferóis, folatos, açúcares, ácidos orgânicos e compostos fenólicos, utilizando métodos cromatográficos e de espetrometria de massa. No caso dos minerais, efeturam-se ainda estudos de bioacessibilidade através de procedimentos de digestão *in vitro*. A bioatividade foi avaliada através da determinação das propriedades:

(i) antioxidantes (atividade captadora de radicais DPPH- 2,2-difenil-1-picril-hidrazilo, poder redutor, inibição da descoloração do β-caroteno e inibição da peroxidação lipídica através do ensaio das espécies reativas do ácido tiobarbitúrico- TBARS);
(ii) citotóxicas em linhas celulares tumorais humanas (ensaio da sulfarrodamina B em MCF-7- carcinoma de mama, NCI-H460- carcinoma de pulmão, HCT 15- carcinoma de cólon, HeLa- carcinoma cervical e HepG2- carcinoma de fígado) e em culturas primárias de células de fígado de porco PLP2;
(iii) antimicrobianas com estirpes ATCC (Coleção de culturas tipo Americana) e bactérias isoladas clinicamente (microdiluição acoplada ao método colorimétrico rápido com cloreto de *p*-iodonitrotetrazólio- INT); e (iv) inibição da produção de biofilme em estripes de bactérias isoladas clinicamente.

Foram também estabelecidas culturas de células e tecidos vegetais com as espécies mencionadas, com o objetivo de otimizar a produção de compostos fenólicos bioativos. Para isso, procedeu-se à esterilização do explante, à germinação das sementes e à aplicação de diferentes concentrações dos fitorreguladores IBA (ácido índolbutírico) e BAP (benzilaminopurina). Da massa vegetal recolhida após crescimento controlado (*F. vesca* foi a única espécie bem-sucedida), fizeram-se novamente os ensaios de caracterização química e avaliação das propriedades bioativas de extratos aquosos (obtidos por infusão e decocção) e hidro-alcoólicos (extração com metanol: água, 80:20, *v/v*).

Finalmente, o extrato mais bioativo (infusão) foi estabilizado por microencapsulação (técnica de atomização/coagulação), tendo-se procedido à caracterização das microesferas obtidas por microscopia ótica (OM – *optical microscopy*), microscopia electrónica de varrimento (SEM – *Scanning electron microscopy*) e espetroscopia de infravermelho com transformada de Fourier (FTIR – Fourier transform infrared spectroscopy), bem como à avaliação da eficiência de encapsulação, esta baseada na quantificação dos compostos identificados na água de coagulação (quercetina-*O*-glucorónido) por cromatografia líquida de alta eficiência (HPLC). O extrato foi posteriormente incorporado, na forma livre e microencapsulada, numa matriz alimentar (gelatina) com vista ao desenvolvimento de nutracêuticos e alimentos funcionais.

Nome científico	*Achillea millefolium* L.	*Fragaria vesca* L.	*Laurus nobilis* L.	*Taraxacum* sect. *Ruderalia*
Nome comum Inglês	Yarrow	Wild strawberry	Laurel	Dendalion
Nome comum Português	Mil-folhas	Morango silvestre	Loureiro	Dente-de-leão
Parte da planta estudada	 Partes vegetativas	 Partes vegetativas  Raízes  Frutos	 Folhas	 Flores  Partes vegetativas
Amostragem	➢Comerciais ➢Silvestres	➢Comerciais ➢Silvestres ➢*In vitro*	➢Comerciais ➢Silvestres	➢Silvestres
Local de recolha silvestres	Cova de Lua, Bragança, Portugal	Serra da Nogueira, Bragança, Portugal	Bragança, Portugal	Bragança, Portugal
Data de recolha silvestres	➢Junho de 2010	➢Julho de 2013	➢ Abril de 2012	➢ Abril de 2012

Figura 1. Descrição das amostras estudadas.

1.3. Organização e estrutura

O documento apresentado é bilingue e está dividido em 7 capítulos distintos, nos quais se abrangem todos os objetivos propostos para o trabalho. Neste capítulo 1 (em português), faz-se a descrição da motivação, dos objetivos da investigação e apresenta-se a organização e estrutura da tese.

O capítulo 2 (em português) apresenta uma revisão do estado da arte no que respeita a cultura *in vitro* como forma de obter plantas enriquecidas em compostos fenólicos, e a microencapsulação como ferramenta para a estabilização de bioativos para fins alimentares.

O capítulo 3 (em inglês) descreve o trabalho experimental associado à caracterização química e nutricional das espécies vegetais: *Achillea millefolium* L. (subcapítulo 3.1), *Fragaria vesca* L. (subcapítulo 3.2), *Laurus nobilis* L. (subcapítulo 3.3) e *Taraxacum* sect. *Ruderalia* (subcapítulo 3.4), bem como a avaliação das propriedades bioativas de extratos aquosos e extratos metanol:água (80:20, *v/v*), obtidos a partir das mesmas. Foca ainda os estudos de digestão *in vitro* (subcapítulo 3.5) para a compreensão da bioacessibilidade de minerais nas amostras mencionadas (plantas secas e extratos aquosos).

O capítulo 4 (em inglês) descreve o estabelecimento de uma cultura *in vitro* de *F. vesca* a partir do seu fruto, com vista à obtenção de clones ricos em compostos fenólicos para utilização na extração de bioativos e desenvolvimento de nutracêuticos.

No capítulo 5 (em inglês) é descrito o trabalho experimental efetuado no tema da microencapsulação do extrato aquoso de *F. vesca*, selecionado uma vez que se apresentou como mais bioativo, entre os estudados. Apresenta ainda a sua aplicação em gelatinas de *k*-carragenina para utilização como alimentos funcionais.

No capítulo 6 (em português) são sintetizadas as conclusões gerais do trabalho desenvolvido, dando destaque à sua contribuição para o desenvolvimento de aplicações alimentares. Adicionalmente apresentam-se as perspetivas futuras do trabalho.

1.4. Plano de trabalho

O trabalho desenvolvido foi organizado em várias fases distintas, como está representado na **Tabela 1**, de forma a alcançar os objetivos mencionados no subcapítulo 1.2 desta tese. De realçar que as tarefas 3 e 4, cultura *in vitro* e microencapsulação, respetivamente, foram desenvolvidas de acordo com os resultados obtidos na tarefa 2.

O trabalho foi desenvolvido em quatro laboratórios de investigação:

- Centro de Investigação de Montanha da Escola Superior Agrária de Bragança do Instituto Politécnico de Bragança;

- Laboratório de Processos de Separação e Reacção da Escola Superior de Tecnologia e Gestão do Instituo Politécnico de Bragança;

- REQUIMTE/LAQV, Laboratório de Bromatologia e Hidrologia do Departamento de Ciências Químicas da Faculdade de Farmácia da Universidade do Porto;

- Departamento de Nutrição e Bromatologia II da Faculdade de Farmácia da Universidade Complutense de Madrid;

A análise dos compostos fenólicos (não antociânicos e antociânicos) foi realizada em colaboração com o departamento de Química Analítica, Nutrição e Bromatologia da Faculdade de Farmácia da Universidade de Salamanca.

Entre o capítulo 3 e 5 desta tese, são apresentados os resultados experimentais na forma de artigos científicos.

Tabela 1. Planificação das tarefas executadas no desenvolvimento desta tese.

Tarefa 1: Recolha e preparação das amostras
Trabalho de campo
➢ Localização e recolha das amostras de plantas propostas para estudo;
➢ Identificação, catalogação e transporte acondicionado para o laboratório;
➢ Identificação botânica e armazenamento em herbário (número *voucher* associado) de um exemplar de cada amostra;
Preparação em laboratório
➢ Congelação e liofilização das amostras;
➢ Preparação dos extratos (aquosos- por infusão ou decocção- e metanol: água 80:20, *v/v*).
Tarefa 2: Caracterização química e avaliação das propriedades bioativas das amostras recolhidas
➢ Composição centesimal: cinzas, proteína, gordura e hidratos de carbono (com discriminação de fibras); valor energético;
➢ Composição individual em ácidos gordos, tocoferóis, açúcares, ácidos orgânicos, minerais (micro e macroelementos) e compostos fenólicos individuais;
➢ Propriedades bioativas: antioxidante, citotóxica, antimicrobiana e inibição da produção de biofilme;
➢ Estudos de bioacessibilidade de minerais.
Tarefa 3: Estabelecimento de uma cultura *in vitro* de *Fragaria vesca* L.
➢ Recolha dos frutos;
➢ Otimização da esterilização, germinação e crescimento;
➢ Recolha de massa vegetal para posterior análise dos parâmetros mencionados na tarefa 2.
Tarefa 4: Microencapsulação de extratos bioativos de *Fragaria vesca* L. e incorporação numa matriz alimentar
Preparação de microesferas ricas em infusão de partes vegetativas de F. vesca
➢ Aplicação da técnica de atomização/coagulação;
➢ Análise à viabilidade das microesferas enriquecidas: OM, SEM, reidratação e eficiência de encapsulação;
Aplicação numa matriz alimentar: gelatina de k-carragenina
➢ Análise da gelatina por MO;
➢ Avaliação da atividade antioxidante e comparação com amostras de gelatina com extrato livre.

Tarefa 5: Elaboração de artigos científicos a partir dos resultados obtidos

- **Artigo I**: Chemical composition of wild and commercial *Achillea millefolium* L. and bioactivity of the methanolic extract, infusion and decoction.
- **Artigo II**: Nutritional parameters of infusions and decoctions obtained from *Fragaria vesca* L. roots and vegetative parts.
- **Artigo II**: Phenolic profile and antioxidant properties of commercial and wild *Fragaria vesca* L. roots: A comparison between hydromethanolic and aqueous extracts.
- **Artigo IV**: Wild *Fragaria vesca* L. fruits: a source of bioactive phytochemicals.
- **Artigo V**: Nutritional and antioxidant contributions of *Laurus nobilis* L. leaves: would be more suitable a wild or a cultivated sample?
- **Artigo VI**: Two-dimensional PCA highlights the differentiated antitumor and antimicrobial activity of methanolic and aqueous extracts of *Laurus nobilis* L. from different origins.
- **Artigo VII**: Nutritional composition, antioxidant activity and phenolic compounds of wild *Taraxacum* sect. *Ruderalia.*
- **Artigo VIII**: Minerals and vitamin B_9 in dried plants vs. infusions: assessing absorption dynamics of minerals by membrane dialysis tandem in vitro digestion.
- **Artigo IX**: Vegetative parts of wild *Fragaria vesca* L.: is *in vitro* culture able to enhance nutritional and bioactive compounds
- **Artigo X**: A bioactive formulation based on *Fragaria vesca* L. vegetative parts: chemical characterization and application in k-carrageenan gelatin.

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2.

Estado da arte

Este capítulo compreende duas temáticas distintas aplicadas à área dos compostos bioativos: por um lado a cultura *in vitro* como ferramenta de produção sustentável em larga escala de compostos fenólicos; por outro lado, a microencapsulação como ferramenta de proteção de bioativos para aplicação em alimentos.

2.1. Explorando a cultura de tecidos vegetais para estimular a produção de compostos fenólicos

2.1.1. Introdução à cultura de células e tecidos vegetais

2.1.1.1. Revisão histórica da cultura de células e tecidos vegetais

A história da cultura de células e tecidos vegetais tem sido extensivamente descrita ao longo dos anos, em livros especializados ou em artigos científicos, mas também em artigos bibliográficos dos seus intervenientes mais importantes como Haberlandt, Gautheret, White, Murashige, Skoog entre outros. Neste sentido, e numa perspetiva de uma visão atualizada sobre a cultura *in vitro*, apresenta-se na **Figura 2** uma breve descrição histórica sobre este tópico, realçando os pontos mais marcantes do desenvolvimento da técnica. Em 1902, Gottlieb Haberlandt propôs a primeira explicação teórica para a cultura *in vitro* de tecidos baseada na totipotência das células vegetais, tendo tentado perceber a funcionalidade e relações estabelecidas entre as células num organismo multicelular. Este estudo foi realizado cultivando células isoladas numa solução nutritiva (Loyola-Vargas & Vázquez-Flota, 2006). Contudo, a primeira cultura de células vegetais só ocorreu em 1922 quando Kotte e Robbins cultivaram raízes e caules de plantas superiores com o intuito de ultrapassar os problemas de esterilização do meio (Kotte, 1922; Robbins, 1922).

A descoberta dos fitorreguladores, também conhecidos por hormonas vegetais, foi também uma etapa que revolucionou o desenvolvimento da cultura *in vitro* de plantas sendo possível, a partir desse momento e de uma certa maneira, controlar os processos fisiológicos envolvidos na germinação e formação de células especializadas, órgãos e tecidos (Roberts, 2012). O primeiro fitorregulador descoberto foi o ácido 3-indolacético (IAA), em 1926, por Went (Hussain et al., 2012). Em 1934-1935, foi desenvolvida a primeira cultura *in vitro* "verdadeira" por Gautheret em associação com White e Nobecourt uma vez que envolveu o estabelecimento de tecido meristemático de *Acer pseudoplatanus* em meio solidificado combinando solução de Knop, glucose, cisteína, IAA e vitaminas levando à diferenciação de tecidos e onde se obteve uma cultura infinita de calli (Gautheret, 1939). A partir desse momento, imensos estudos de investigação em diferentes plantas, órgãos e tecidos foram direcionados para o teste de diferentes combinações de soluções nutritivas. As décadas entre 1940 e 1960 são apontadas como das mais importantes para o desenvolvimento da grande maioria das técnicas para cultura *in vitro* ainda hoje usadas. Durante esse período houve também muito avanço no campo dos fitorreguladores, tendo-se descoberto a cinetina em 1955 como hormona da divisão celular o que levou os investigadores Skoog e Miller, em 1957, à descoberta do controlo hormonal para formação

de determinados órgãos em cultura ajustando simplesmente a concentração/rácio de auxinas e citoquinas no meio (Skoog & Miller, 1957).

Mas a descoberta mais importante foi conseguida por Murashige e Skoog, em 1962, aquando do desenvolvimento do muito conhecido meio MS (Murashige Skoog) para cultura de células de tabaco que consiste numa alta concentração de sais, mas baixa concentração de azoto, macro e micronutrientes, uma fonte de carbono (p. ex.: sacarose), vitaminas do complexo B e também fitorreguladores (Murashige & Skoog, 1962). O que estes investigadores alcançaram pelo desenvolvimento do meio MS foi a combinação de todos os requisitos nutricionais para um grande grupo de plantas, permitindo que este meio seja ainda hoje usado por muitos investigadores nos seus estudos sobre cultura *in vitro* de plantas (Loyola-Vargas & Vázquez-Flota, 2006).

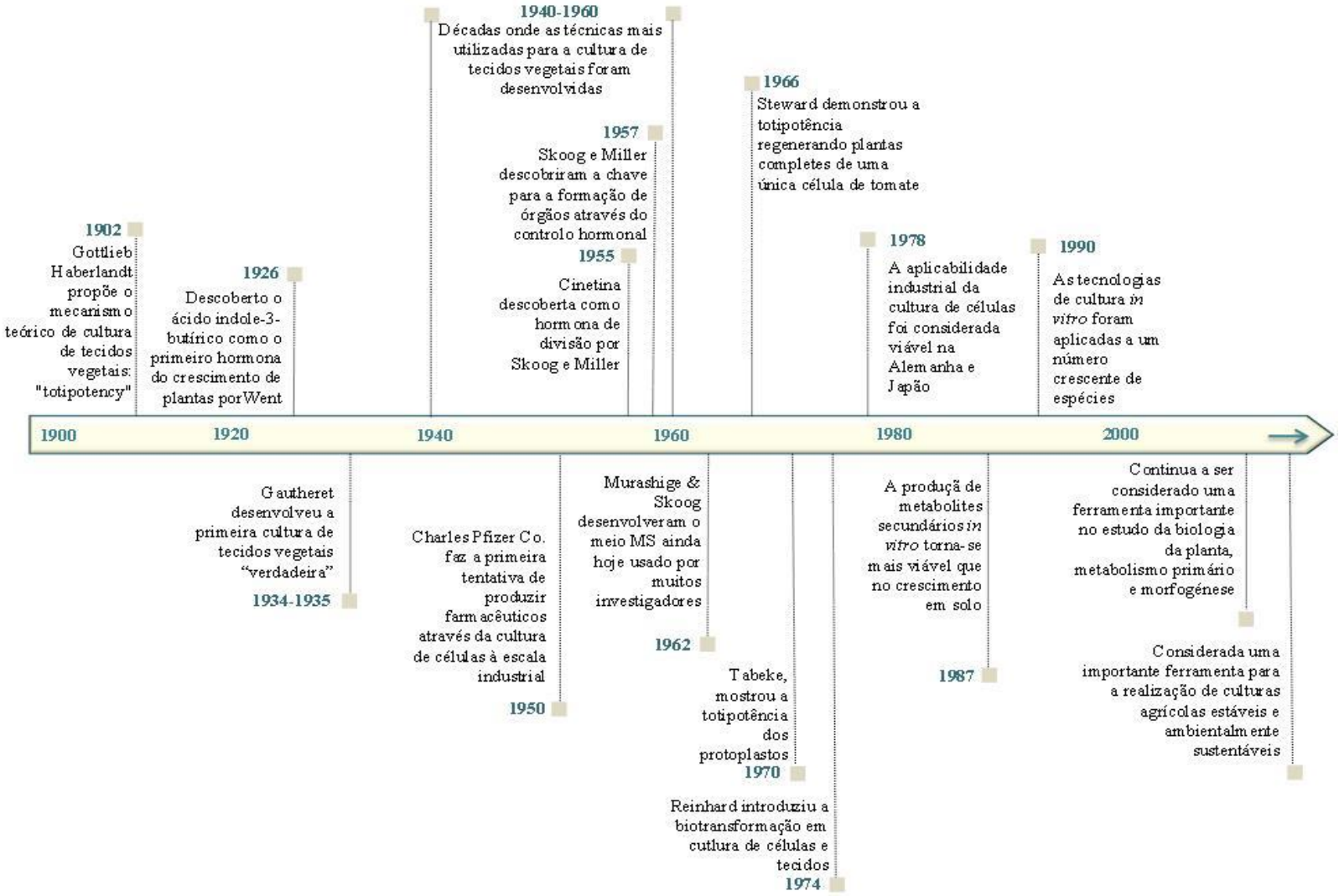


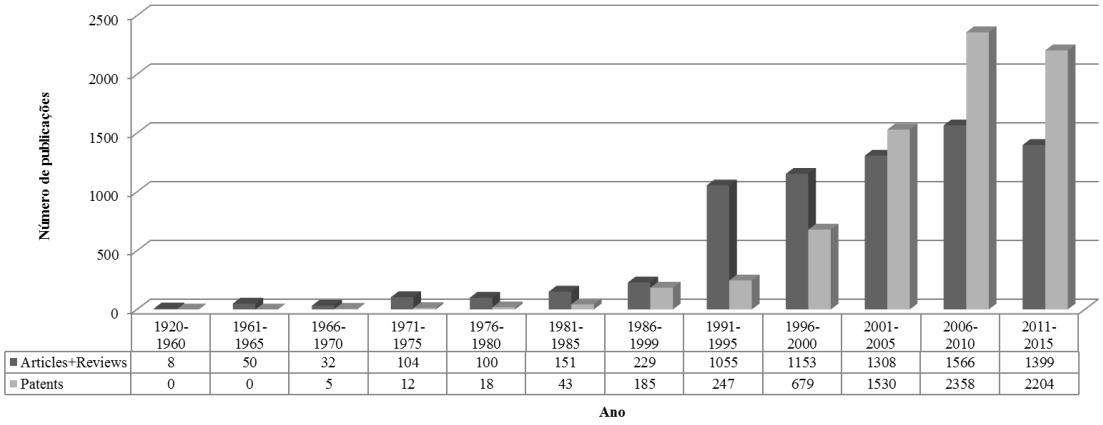
Figura 2. Etapas históricas mais importantes no desenvolvimento de técnicas de cultura *in vitro* e produção de metabolitos (Dias et al., 2016).

Nos anos seguintes, muitos investigadores prosseguiram com a investigação do papel dos fitorregulares do desenvolvimento de plantas *in vitro* (Loyola-Vargas & Vázquez-Flota, 2006; Hussain et al., 2012), e a totipotência das células vegetais foi demonstrada tanto em partes cada vez mais pequenas e desdiferenciadas da planta, como em células individuais por Stewart, em 1966, e em protoplastos por Tabeke, em 1970 (Loyola-Vargas & Vázquez-Flota, 2006).

Em relação à utilização da cultura de tecidos vegetais para a produção de metabolitos secundários com grande interesse fitoquímico, a primeira tentativa ocorreu em 1950 pela Companhia Charles Pfizer com a intenção de produzir compostos fitofármacos para a indústria farmacêutica em grande escala, especialmente penicilina, recorrendo a um bolor extraído de melões crescidos *in vitro* (Lombardino, 2010). No entanto, a aplicabilidade industrial da cultura de células para a produção de metabolitos secundários foi somente considerável viável em 1978 na Alemanha e Japão (Loyola-Vargas & Vázquez-Flota, 2006). Apenas uma década depois, em 1987, a produção destes metabolitos recorrendo a técnicas *in vitro* ultrapassou a produção em plantas crescidas em solo, com base em resultados de mais de 30 sistemas de cultura de células diferentes, tendo sido considerado um processo economicamente viável para a produção dos mesmos (Savangikar, 2004; Loyola-Vargas & Vázquez-Flota, 2006). Os protocolos de imobilização e técnicas de “scale-up” permitiram o desenvolvimento de sistemas de produção de metabolitos *in vitro* que são funcionais a nível comercial, sendo usados para a produção de vacinas e proteínas (Kintzios, 2008). Uma das maiores histórias de sucesso é a produção de taxol e ácido rosmarínico para utilização comercial pelas suas propriedades quimioterapêuticas e atividade antioxidante, respetivamente (Kintzios, 2008). A expansão da cultura de células e tecidos vegetais continuou, tendo sido aplicada a um número crescente de espécies de plantas e em várias áreas de aplicação. No entanto, para os investigadores continuou a ser uma ferramenta muito importante para o estudo da morfogénese, metabolismo primário e outros processos fisiológicos (Collin, 2001; Smetanska, 2008).

Apesar do conceito de cultura de células vegetais ter aparecido no início do século XX, os primeiros artigos publicados sobre o tema só apareceram em meados da década de 20 (**Figura 3**) e até à década de 60 não houve qualquer progresso por parte da academia em termos de resultados publicáveis. No entanto, e como foi dito anteriormente, foi a partir da década de 60 que ocorreu o maior impulso na cultura de células e tecidos vegetais, com o desenvolvimento de novas técnicas e meios de cultura, mas também com a descoberta da importância fisiológica dos fitorreguladores. Como pode ser observado na **Figura 3**, desde o início da década de 60 houve um notável crescimento no número de artigos relativamente a esta matéria. Em termos de indústria, até aos meados da década de 80 o seu interesse não era significativo quando comparado com os resultados publicados pela academia. No

entanto, o número de patentes relacionados com a cultura de células ultrapassou atualmente o número de trabalhos publicados em investigação.



	1920-1960	1961-1965	1966-1970	1971-1975	1976-1980	1981-1985	1986-1999	1991-1995	1996-2000	2001-2005	2006-2010	2011-2015
Articles+Reviews	8	50	32	104	100	151	229	1055	1153	1308	1566	1399
Patents	0	0	5	12	18	43	185	247	679	1530	2358	2204

Figura 3. Número de artigos de investigação e revisão, e patentes publicadas no período compreendido entre 1920 e 2015 relativamente à cultura de células e tecidos vegetais (dados obtidos no web of science, Fevereiro de 2015; palavras-chave: “cell and tissue culture” e “plant”) (Dias et al., 2016).

O principal objetivo deste capítulo é realçar as vantagens da produção de compostos fenólicos (incluindo antocianinas) em cultura de células e tecidos vegetais, uma vez que de todos os compostos bioativos referidos no sub-capítulo 2.1.2, os compostos fenólicos são os que nos despertam maior interesse e para o qual o grupo de investigação está mais direcionado. Será feita uma apreciação sobre o valor acrescentado deste tipo de compostos e também das vias biossintéticas envolvidas na sua produção. Vários extratos e compostos produzidos por técnicas de cultura *in vitro* serão também enumerados, assim como as técnicas de elicitação mais usadas para a produção dos mesmos, realçando os também os próprios compostos fenólicos como elicitores.

2.1.1.2. Benefícios do uso da cultura de células e tecidos vegetais

A Organização Mundial de Saúde (“*World Health Organization*”- WHO) estima que hoje em dia 80% da população mundial ainda depende da fitoterapia para obtenção de cuidados básicos de saúde, usando as plantas aromáticas e medicinais numa base diária para obter esses mesmos cuidados. Para além disso, dois terços dos medicamentos anticancerígenos e contra doenças infecciosas existentes hoje no mercado são também derivados de plantas (Peter et al., 2005; Kolewe et al., 2008). Com a procura incessante do mercado por produtos derivados de matrizes naturais surge, assim, uma preocupação ambiental relativamente à perda de populações de plantas, diversidade genética, degradação de habitats e, em último caso, extinção de espécies (Roberto et al., 2011).

A cultura de células e tecidos vegetais surge assim como uma técnica biotecnológica viável para a produção de compostos bioativos que podem ser usados nas mais diversas áreas e tendo sobretudo em vista um esforço adicional para a conservação sustentável e utilização racional da biodiversidade (Karuppusamy, 2009). Em 1994, a Organização das Nações Unidas para Alimentação e Agricultura ("*Food and Agricultural Organization*"- FAO) endossou a técnica de cultura de células e tecidos vegetais, processo para a produção de compostos naturais, para fins alimentares (Anand, 2010; Roberto et al., 2011). Foi publicado em 2002 pela FAO em conjunto com a IAEA (Divisão de técnicas nucleares para a alimentação e agricultura –"*Division of Nuclear Techniques in Food and Agriculture*") um relatório no qual abordam a temática da cultura *in vitro* para produção de compostos bioativos com valor acrescentado e de que forma os investigadores e indústria de países emergentes o podem fazer da maneira mais economicamente possível (FAO/IAEA, 2002). Murthy et al. (2015), fazem uma avaliação sobre a segurança dos ingredientes alimentares derivados da cultura de células e tecidos vegetais e propõem uma série de protocolos para avaliação de uma possível toxicidade destes produtos, mas também para avaliar potenciais bioatividades presentes.

A definição mais abrangente da cultura de células e tecidos é a manipulação de células ou órgãos em condições asséticas, crescidas num meio de cultura sob condições controladas de luz, humidade e temperatura (Smetanska, 2008). Este sistema de produção controlada permite o aumento da uniformidade e da padronização dos extratos, assim como das concentrações dos compostos desejados, mantendo as mesmas características genéticas nos clones de maior produção (Chaturvedi et al., 2007).

Em teoria, a cultura de células e tecidos vegetais pode ser aplicada a qualquer planta, pois cada célula vegetal apresenta no seu genoma o conjunto de genes necessários para manter as funções num meio artificial, incluindo o metabolismo secundário e a totipotência. No entanto, quando se pensa numa aplicação, principalmente industrial, a viabilidade do processo é muito importante, mas também a competitividade do método face a outros já existentes (Verpoorte et al., 1999). É uma técnica muito apelativa para os investigadores e indústria porque na sua essência colmata dois problemas das plantas crescidas em solo: o crescimento muito lento e dependente das condições climatéricas e os baixos rendimentos de produção de metabolitos secundários. Células especializadas, como rebentos e raízes, crescidas *in vitro* podem apresentar um perfil metabólico semelhante às plantas nativas, podendo também haver produção em células não especializadas (Kolewe et al., 2008). A combinação entre processos de engenharia biotecnológica e bioquímica direcionada levou a uma melhoria significativa nos rendimentos de produção (Kolewe et al., 2008) e tornou a cultura *in vitro* o método de eleição para a produção de compostos bioativos (Zhou & Wu, 2006).

Há uma série de vantagens relacionadas com a produção de compostos bioativos *in vitro*: condições de produção optimizadas e controladas; controlo do produto final; engenharia genética para a escolha dos melhores clones; produção de compostos puros; melhoria do efeito nutricional da planta produzida; diminuição de compostos indesejados; não é necessário o uso de pesticidas e herbicidas; síntese de novos compostos e não estar dependente de condições climatéricas e geográficas (Verpoorte et al., 1999; Chattopadhyay et al., 2002).

Há também uma procura no mercado por corantes de origem natural em substituição de compostos sintéticos com elevada toxicidade. A cultura *in vitro* é também muitas vezes usada para a produção deste tipo de compostos, como as antocianinas, não ocorrendo a degradação dos compostos causada pelo armazenamento ou por processos de extração (Zhang & Furusaki, 1999). As culturas meristemáticas podem também ser usadas para a produção e, principalmente, multiplicação de plantas com elevado potencial bioativo, uma vez que estas se podem desdiferenciar em novas células, órgãos e tecidos (Lee et al., 2010). A marcação dos compostos bioativos por rádio é também muitas vezes usada em cultura de células para estudo das vias metabólicas de produção de determinados compostos (Anand, 2010).

Em última análise, a grande vantagem da técnica de cultura de células e tecidos vegetais é poder providenciar uma produção contínua, sustentável, económica e fiável de compostos naturais, independentemente das condições geo-climatéricas sobre um regime de microambiente altamente controlado (Karuppusamy, 2009; Anand, 2010).

2.1.2. Compostos fenólicos e elicitores

2.1.2.1. Valor acrescentado das plantas ricas em compostos fenólicos

As propriedades dos compostos fenólicos são muito reconhecidas, havendo um número incontável de artigos de investigação e revisão sobre as suas caraterísticas biológicas mas também bioativas. Num artigo de revisão, Quideau et al. (2011) fizeram o levantamento das propriedades químicas e estruturais de toda a classe de compostos fenólicos correlacionando-as com as suas propriedades biológicas, e de que forma é que são expressas quando estes compostos são ingeridos numa base diária, em frutas e vegetais, mas também em bebidas como o vinho tinto e mesmo no chocolate. Concluíram que, apesar da sua fraca solubilidade e biodisponibilidade, estes compostos podem ter efeitos benéficos a longo prazo quando consumidos numa base diária e que a síntese química, ao serviço da comunidade académica e industrial, providencia análogos destes compostos que podem ser introduzidos na alimentação. Os compostos fenólicos

representam hoje em dia a família de compostos mais estudados em todo o mundo pelas suas propriedades bioativas, ocorrendo naturalmente nas plantas e apresentam uma enorme diversidade estrutural e química. Muitos estudos estão ainda direcionados para a sua estrutura química e biológica, bem como para as suas vias biossintéticas (p. ex.: enzimas envolvidas, genes e proteínas) (Boudet, 2007; Cohen & Kennedy, 2010). Por todas estas razões, os compostos fenólicos tornaram-se um alvo apetecível para a técnica de cultura de células e tecidos na procura de compostos com propriedades antioxidantes *in vitro* uma vez que, sendo produtos do metabolismo secundário, são produzidos e excretados pelas plantas em condições de stresse que a cultura *in vitro* providencia (Matkowski, 2008). Tem também sido dado um ênfase especial à produção de antocianinas *in vitro* pelas suas reconhecidas propriedades bioativas e também porque estes pigmentos são facilmente degradáveis e polimerizados com alterações de pH (Zhang & Furusaki, 1999). Para além das suas propriedades antioxidantes, os compostos fenólicos têm também interessado aos investigadores pelas suas propriedades anticancerígenas, antifibrinogénicas e também por constituírem conservantes naturais (Quideau et al., 2011; Carocho & Ferreira, 2013).

2.1.2.2. Vias biossintéticas de compostos fenólicos em plantas e a influência da elicitação

Nas plantas uma quantidade significativa de carbono e energia são direcionados à produção de moléculas cuja função ainda não é totalmente conhecida. O metabolismo central nas células vegetais é baseado nas vias respiratórias, glicólise e ciclo do ácido cítrico, onde é produzida a vasta maioria das moléculas e compostos envolvidos na sobrevivência e defesa das plantas (Lobo & Lourenço, 2007). Os compostos fenólicos são referenciados como metabolitos secundários uma vez que não estão diretamente relacionados com as funções de crescimento e desenvolvimento do tecido vegetal, e são normalmente encontrados em tecidos e órgãos específicos, e em estágios de desenvolvimento específicos (Buchanan & Jones, 2000).

Existem centenas de compostos fenólicos diferentes em termos de estrutura que, por consequência, apresentam diversas atividades biológicas distintas, dependendo também da concentração em que são consumidas (Karakaya, 2004; Quideau et al., 2011).

A estrutura base dos compostos fenólicos é um anel benzénico com substituintes hidroxilo (Cohen & Kennedy, 2010). Na **Figura 4**, está representado um sumário da altamente complexa via metabólica dos fenilpropanóides e são descritos alguns dos mais importantes passos para a formação de alguns compostos fenólicos. A via mais importante na biossíntese dos compostos fenólicos é a via do ácido xiquímico no qual uma molécula de ácido fosfoenolpirúvico (PEP) derivado da glicólise e a eritrose-4-fosfato derivada da via das

pentoses fosfato resultam na formação de um açúcar de sete carbonos denominado de DAHP (3-deoxi-O-arabino-heptulosonato fosfato), sendo depois ciclizado e reduzido para formar o xiquímico. A formação do xiquímico é um ponto crítico na formação de compostos fenólicos. É importante notar que a via metabólica do ácido xiquimco está também envolvida na formação de proteínas, metabolitos primários com funções essenciais nos tecidos das plantas e dessa maneira compete diretamente com a formação dos compostos fenólicos (Karakaya, 2004; Cohen & Kennedy, 2010). A partir desta estrutura ocorre a formação dos ácidos fenólicos (ex. ácido protocatecuico e ácido elágico), fenóis simples possuindo apenas um grupo carboxílico e servindo de precursores de outros compostos (Cohen and Kennedy, 2010). Podendo também levar à formação de aminoácidos aromáticos, fenilalanina, tirosina e triptofano, começando a via metabólica dos fenilpropanóides a partir deste momento. A biossíntese dos aminoácidos aromáticos é um exemplo de mecanismo de feedback, significando que uma maior produção direcionada para o triptofano vai induzir um fluxo de carbono para a produção de fenilalanina e tirosina (Verpoorte et al., 1999). Metabolicamente isto é muito interessante, uma vez que a produção de fenólicos mais complexos começa com a desaminação da fenilalanina em ácido cinâmico (diretamente para a produção de cumarinas) e depois a conversão em ácido *p*-cumárico (também derivado da tirosina). Da produção de ácido *p*-cumárico pode levar à produção de ácidos hidroxicinâmicos como o ácido cafeico, sendo este isto último convertido na sua forma álcool que depois com a forma álcool do ácido p-cumárico podem levar à produção de lenhina. Pela ação das enzimas CHS (chalconas sintetase), CI (chalconas isomerase) e F3H (flavanona-3-hidroxilase), o ácido *p*-cumárico é então convertido em flavonoides (ex. quercetina) e flavan-3-óis (direcionado para a produção de proantocianidinas e antocianinas).

Apesar de as plantas produzirem naturalmente compostos fenólicos quando colocadas *in vitro*, existem muitas situações onde é necessário melhorar essa produção. Devido à breve fase estacionária que as plantas *in vitro* apresentam, os metabolitos secundários, em geral, produzidos com baixos rendimentos (inibição da ação das enzimas, normalmente apresentada em plantas maturas) (Michael and John, 1985). A elicitação é usada para aumentar a produção e cumulação de metabolitos secundários através de sistemas de produção *in vitro*, despoletando respostas morfológicas e fisiológicas. Esta estimulação ocorre em resposta a estímulos de stress de compostos sinal que ativam os mecanismos de defesa das plantas (Rea et al., 2011). A elicitação química é conseguida através de fitorreguladores, moléculas sinalizadoras e pela adição de moléculas precursoras. A elicitação física é feita através de irradiação UV, pressão, campos elétricos, concentração de metais pesados, pH e temperatura. Microorganismos, fungos e bactérias, podem funcionar como elicitores biológicos (Mewis et al., 2011; Baenas et al., 2014). A **Figura 8** mostra também alguns pontos onde a elicitação pode ser usada para aumentar a

produção de compostos fenólicos, por exemplo, a enzima que cataliza a reação de desaminação da fenilalanina em ácido cinâmico é PAL (fenilalanina amónia-liase). A atividade desta enzima é estimulada por radiação vermelha e UV (Boudet, 2007), estando por isso presente na cultura *in vitro* como elicitor físico. Há uma ligação ecológica entre a elicitação e a produção de certos tipos de compostos fenólicos dependendo do propósito destes compostos na cultura. Por exemplo, a produção de antocianinas é altamente influenciada pela quantidade de luz (elicitação física) que incide nos tecidos vegetais, uma vez que estes compostos servem como absorvente de luz e por isso protegem as células dos seus efeitos adversos (Dixon & Paiva, 1995; Zhang & Furusaki, 1999). A produção destes compostos é também conseguida por outro tipo de elicitores físicos, como a temperatura e pH, mas também por adição de percursores e optimização do meio de cultura (elicitação química). A produção de cumarinas, por exemplo, é conseguida através da elicitação biológica, usando microorganismos que induzem a sua produção, uma vez que este tipo de compostos estão relacionados com a proteção dos tecidos vegetais contra ataques de patogénicos.

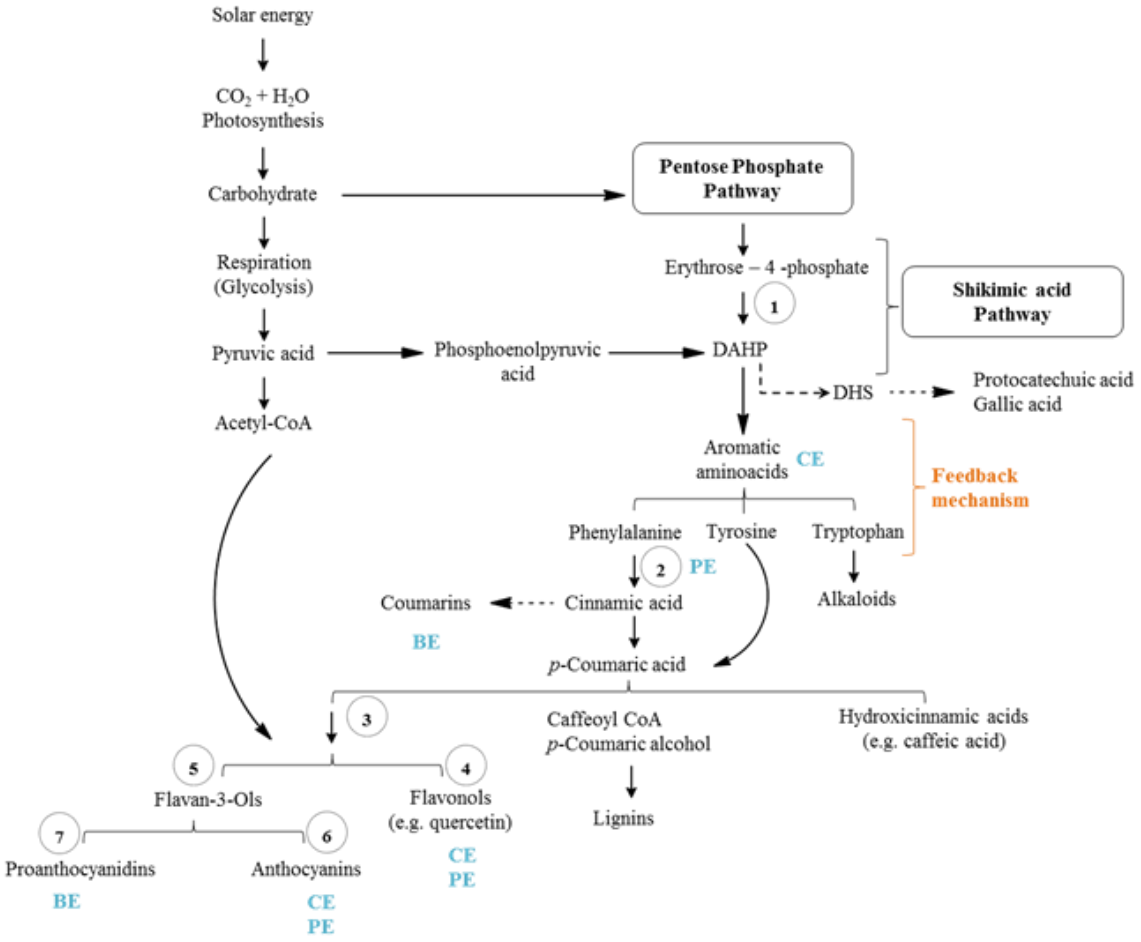


Figura 4. Via biossintética de alguns compostos fenólicos e a influência da elicitação (Dias et al., 2016).

CO_2- Dióxido de carbono; H_2O- Água; Acetil- CoA-AcetilCoenzima A; DAHP- 3-Deoxi-O-arabino-heptulosonato fosfato; DHS- 3-Dehidroquinato; BE- Elicitação Biológica; CE- Elicitação química; PE- Elicitação física; As enzimas envolvidas na biossíntese estão marcadas com formas arredondadas a preto tracejado: 1- DAHP sintase (3-Deoxi-O-arabino-heptulosonato fosfato); 2- PAL (Fenilalanina Amónia-liase); 3- CHS (Chalconas sintase), CHI (Chalconas isomerase), F3H (Flavanona-3-hidroxilase); 4- FLS (Flavonol sintase); 5- LAR (Leucoantocianidinas reductase); 6- LDOX (Leucoantocianidina dioxigenase).

2.1.3. Incremento na produção de compostos fenólicos

2.1.3.1. Produção de compostos fenólicos por técnicas de cultura in vitro de plantas

Há inúmeros estudos sobre a produção de metabolitos secundários e cultura *in vitro* de plantas e imensas revisões que compilam muita dessa informação *(*Zhang & Furusaki, 1999; Chattopadhyay et al., 2002; Karuppusamy, 2009; Matkowski, 2008). No entanto, a grande maioria da informação necessita de ser atualizada e focalizada somente na produção de compostos fenólicos. Na **Tabela 2**, estão descritos os compostos fenólicos (extratos fenólicos e individuais, incluindo antocianinas) produzidos em cultura de células vegetais, descrevendo a origem dos mesmos e o processo de extração dos compostos. A produção de extratos fenólicos é o objetivo da grande maioria dos estudos revistos (Andarwulan & Shetty, 1999; Lozovaya et al., 2000; Santos-Gomes et al., 2003; Gális et al., 2004; Lozovaya et al., 2006; Kouakou et al., 2007; Bairu et al., 2011; Cui et al., 2011;

Krzyzanowska et al., 2011; Palacio et al., 2012; Szopa et al., 2013; Siu et al., 2014; Szopa &Ekiert, 2014; Yildirim & Turker, 2014). No entanto, alguns desses trabalhos focalizam-se também no estudo de propriedades bioativas desses extratos fenólicos, nomeadamente propriedades antioxidantes (Grzegorczyk et al., 2007; Hakkim et al., 2007; Kovatcheva-Apostolova et al., 2008; Hussein et al., 2010; Amoo et al., 2012; Giri et al., 2012; Khateeb et al., 2012; Barros et al., 2013; Bhagya & Chandrashekar, 2013; Chaniany et al., 2013; Madhu, 2013; Goyali et al., 2014; Lugato et al., 2014; Piątczak et al., 2014; Valdez-Tapia et al., 2014), antimicrobianas (Hussein et al., 2010; Ncube te al., 2011; Zhao et al., 2011; Khateeb et al., 2012) e mesmo citotóxicas (Skoríc et al., 2012).

Tabela 2. Extratos fenólicos e compostos fenólicos individuais produzidos por cultura de tecidos vegetais (Dias et al., 2016)

Composto/extrato bioativo	Origem	Solvente de extração	Referência
Antocianinas	*Eugenia myrtifolia* Sims (rebentos)		Longo et al. 2007
Extratos bioativos	*Satureja hortensis* L. (calli)	Metanol	Güllüce et al., 2003
Glucoiridóides	*Penstemon serrulatus* Menz. (calli)	Etanol	Bazylak et al., 1996
Compostos fenólicos individuais	*Helichrysum aureonitens* L. Moench (calli)	Água:Etanol (5:95, v/v)	Ziaratnia et al. 2009
	Mirabilis jalapa L. (calli)	Diclorometano:Metanol (50:50, *v/v*)	Yang et al. 2001
	Ocimum americanum L., var. pilosum (rebentos)	Extratos alcoólicos	Rady & Nazif, 2005
	Psoralea corylifolia L. (calli)	Ácido sulfúrico	Shinde et al. 2010
	Rauwolfia serpentina Benth. ex Kurz (células)	Metanol	Schroeder et al. 1996
	Salvia miltiorrhiza Bunge (raízes tansgénicas)	Metanol	Chen te al., 1999
	Scutellaria baicalensis Georgi (raízes tansgénicas)	Metanol	Nishikaw et al. 1999
Ácidos fenólicos	*Eryngium planum* L. (raízes e rebentos)	Água:Metanol (50:50, v/v)	Thiem et al., 2013
	Schisandra chinensis (Turcz.) Baill.(calli)	Metanol	Szopa & Ekiert 2012
	Theobroma cacao L. (estaminódios/anteras)	Água:Metanol (20:80, *v/v*)	Alemanno et al. 2003
Compostos fenólicos	*Aloe arborescens* Mill rebentos	Água:Metanol (50:50, *v/v*)	Amoo et al., 2012
	Aronia melanocarpa (Michx.) Elliott (rebentos e calli)	Metanol	Szopa & Ekiert, 2014
	Aronia melanocarpa (Michx.) Elliott (rebentos e calli)	Metanol	Szopa et al., 2013
	Brassica nigra L. (calli)	Metanol	Hussein et al., 2010
	Castilleja tenuiflora Benth. (rebentos)	Metanol	Valdez-Tapia et al., 2014
	Cichorium pumilum Jacq. (calli)	Água:Metanol (5:95, *v/v*)	Khateeb et al. 2012
	Cistus creticus subsp.creticus L. (rebentos e raízes)	Água:Etanol (3:97, *v/v*)	Skoríc et al. 2012
	Clitorea ternatea L.(rebentos)	Etanol	Madhu 2013
	Coriandrum sativum L.(partes vegetativas)	Água:Metanol (20:80, *v/v*)	Barros et al. 2012
	Daucus carota L. (raízes transgénicas)	Água:Metanol (50:50, v/v)	Sircar et al., 2007
	Fagopyrum tataricum Gaertn. (calli)		Lozovaya et al., 2000
	Fragaria vesca L. (folhas e calli)	Metanol	Yildirim & Turker, 2014
	Gossypium hirsutum L. (calli)	Metanol	Kouakou et al. 2007
	Habenaria edgeworthii Hook. f. ex. Collett (calli)	Água:Metanol (20:80, *v/v*)	Giri et al. 2012
	Hypericum perforatum L. (raízes adventícias)	Água:Metanol (20:80, *v/v*)	Cui et al., 2011
	Hypericum polyanthemum (partes aéreas)	Metanol	Nunes et al., 2009
	Hypericum rumeliacum Boiss. (rebentos)	Metanol	Danova et al. 2010
	Hypericum ternum A. St. Hil. (partes aéreas)	Metanol	Pinhatti et al. 2010
	*Juglans regia*L. (micro-rebentos)	Água:Metanol (20:80, *v/v*)	Cheniany et al., 2013
	Justicia gendarussa Burm. f. (caules, folhas e calli)	Etanol, metanol e éter	Bhagya & Chandrashekar, 2013
	Larrea divaricata Cav. (calli)	Água:Metanol (5:95, v/v)	Palacio et al. 2012
	Lavandula vera DC	Água:Etanol (60:40, *v/v*)	Kovatcheva-Apostolova et al. 2008
	Melissa officinalis L. (partes aéreas)	Água	Barros et al., 2013
	Mentha longifolia (L.) Huds. (calli e células)	Água:Metanol (30:70, v/v)	Krzyzanowska et al., 2011
	Mentha piperita L. (calli e células)	Água:Metanol (30:70, v/v)	Krzyzanowska et al., 2011
	Nicotiana tabacum L.cv. Samsun (calli e rebentos)	Água:Metanol (20:80, *v/v*)	Gális et al., 2004

	Ocimum sanctum L. (calli)	Água:Metanol (20:80, v/v)	Hakkim et al., 2007
	Passiflora alata Curtis (folhas)	Água:Etanol misturas	Lugato et al., 2014
	Pimpinella anisum L. (raízes)	Água:Etanol (5:95, *v/v*)	Andarwulan & Shetty, 1999
	Rehmannia glutinosa Libosch (folhas e raízes)	Metanol	Piątczak et al., 2014
	Rosa damascena Mill.	Água:Etanol (60:40, *v/v*)	Kovatcheva-Apostolova et al. 2008
	Salvia miltiorrhiza Bunge (raízes transgénicas)	Água:Etanol (5:95, v/v)	Zhao et al., 2011
	Salvia miltiorrhiza L. (raízes transgénicas)	Tampão fosfato (75mM, pH=7)	Siu et al., 2014
	Salvia officinalis L. (rebentos e raízes)	Metanol ou acetona	Grzegorczyk et al 2007
	Salvia officinalis L. (calli e células)	Acetona	Santos-Gomes et al. 2003
	Solidago graminifolia L. (plântulas e calli)	Metanol	Thiem et al., 2011
	Solidago virgaurea L. (plântulas e calli)	Metanol	Thiem et al., 2011
	Tulbaghia violacea Harv. (partes aéreas)	Água:Metanol (50:50, v/v)	Ncube et al. 2011
	Vaccinium angustifolium Ait. (folhas)	Água:Acetona:Ácido fórmico (20:80:0.1 *v/v/%*)	Goyali et al., 2014
	Zea mays L. (calli)		Lozovaya et al., 2000
	Zea mays L. (calli)		Lozovaya et al., 2006
Compostos fenólicos e antocianinas	*Ipomoea batatas* L. cv Ayamurasaki (calli)	Ácido acético 16%	Konczak-Islam et al., 2003; Konczak-Islam et al., 2005
Compostos fenólicos em extrato de betalaínas	*Beta vulgaris* cv. Detroit Dark Red (raízes transgénicas)	Água:Etanol (30:70 *v/v*)	Georgiev et al. 2010
Compostos fenólicos, flavanois	*Taxus baccata* L. (calli)	Água:Etanol (30:70 *v/v*)	Dubravina et al. 2005
	Taxus canadensis Marsh. (calli)	Água:Etanol (30:70 *v/v*)	Dubravina et al. 2005
Compostos fenólicos, galotaninos, iridóides	*Harpagophytum procumbens* (Burch.) DC. ex Meisn (plântulas e calli)	Água:Metanol (50:50, *v/v*)	Bairu et al. 2011
Compostos fenólicos tetra-hidroprotoberberinas	*C. ochotensis* var. raddeana (calli)		Iwasa et al. 2010
	M. cordata R.Br. (calli)		Iwasa et al. 2010
	N. domestica Thunb. (calli)		Iwasa et al. 2010

Os extratos antociânicos são também muito apelativos para a produção de metabolitos secundários em estudos de cultura *in vitro*. São pigmentos naturalmente produzidos pelas plantas, frutos e vegetais com um grande potencial antioxidante, apresentando também outras bioatividades tais como antitumoral, anti-inflamatória e antimutagénica (Kong et al., 2003). Konczak-Islam et al. (2003) e Konczak-Islam et al. (2005) obtiveram concentrações elevadas de antocianinas acetiladas em *calli* de *Ipomoea batatas* L. cv Ayamurasaki após transferência para um meio próprio para a produção de antocianinas. Neste mesmo estudo, os ácidos clorogénico e cafeico foram também identificados como os compostos fenólicos maioritários. Por outro lado, Barros et al. (2012) detetaram a produção de antocianinas num clone obtido de partes vegetativas de *Coriandrum sativum* L. no mesmo meio MS onde os restantes clones foram também produzidos, sugerindo que a produção de antocianinas pode ser afetada pelas condições ambientais ou stresse fisiológico da cultura *in vitro*. Longo et al. (2007) focaram-se somente na produção e caraterização de antocianinas em rebentos de *Eugenia myrtifolia* Sims, observando que esta planta produz somente uma forma molecular de malvidina, uma das antocianinas mais comuns em plantas superiores (Kong et al., 2003), podendo ser usada como modelo para o estudo das vias biossintéticas destes compostos.

As betalaínas são também pigmentos usados como corantes alimentares, apresentando um elevado potencial antioxidante devido à presença de grupos hidroxilo fenólicos na sua estrutura. Georgiev et al. (2010) estudaram a composição fenólica em extratos de betalaínas excretados para o meio de cultura por raízes transgénicas de *Beta vulgaris* cv. Detroit Dark Red, observando que estes extratos apresentavam maior atividade antioxidante que o material vegetal inicial, concluindo sobre a existência de efeitos sinergísticos entre as betalaínas e os concomitantes compostos fenólicos. Dubravina et al. (2005) focaram-se na produção de compostos fenólicos flavanois em *calli* de *Taxus baccata* L. e *T. Canadensis* Marsh. durante o período de um ano, notando um aumento significiativo destes compostos em condições que mimetizam o verão, e também durante a diferenciação dos tecidos. Isawa et al. (2010) obtiveram compostos fenólicos tetra-hidroprotoberberinas a partir de tecido de calli de *Corydalis ochotensis* var. raddeane, *Macleaya cordata* R.Br e *Nandina domestica* Thunb, importantes pela sua atividade antimalária e, por isso, apelativos para a indústria farmacêutica. Os ácidos fenólicos são também um grupo de compostos que demonstram uma alta potencialidade fitoquímica devido às suas caraterísticas biológicas. Thiem et al. (2013) estudaram a produção de ácidos fenólicos em raízes transgénicas e rebentos de *Eryngium planum* L., observando níveis elevados de ácidos cafeico e clorogénicos, mas sobretudo, ácido rosmarínico excretado pelas raízes (procedimento de extração facilitado). Szopa & Ekiert (2012) encontraram também níveis elevados de ácidos *p*-cumárico, *p*-hidroxibenzóico, protocatéquico, salicílico e siríngico em *calli* de *Schisandra*

chinensis (Turcz.) Baill., aumentando o valor fitoquímico desta planta. Alemanno et al. (2003) estudaram estaminóides e anteras de *Theobroma cacao* L. descobrindo que a cultura *in vitro* é uma técnica fiável para a manutenção e multiplicação de clones de alta produção desta planta, identificado também três ácidos fenólicos derivados de amidas de ácidos hidroxicinâmicos, nunca antes identificados em tecidos originais.

Todos os estudos acima mencionados referem-se a extratos fenólicos. No entanto, há já muitos estudos que estão já focalizados para a produção e extração de compostos fenólicos individuais que possam ter caraterísticas bioativas interessantes. Na **Figura 5** estão representados esquematicamente alguns compostos fenólicos individuais produzidos em cultura de células vegetais. Como foi dito anteriormente, os ácidos fenólicos são um grupo de compostos que incitam os investigadores pelas suas propriedades bioativas. A maioria dos estudos são direcionados para a produção destes compostos. Chen et al. (1999) estudaram a produção de ácido litospémico B (**3a**) e ácido rosmarínico (**3b**) em raízes transgénicas de *Salvia miltiorrhiza* Bunge, enquanto Rady & Nazif (2005) produziram ácido rosmarínico em rebentos de *Ocimum americanum* L. var pilosum, pelo seu potencial bioativo. O ácido *o*-coumárico glicosilado (**3c**) e o ácido cinâmico glicosilado (**3d**) foram ambos produzidos numa suspensão celular de *Rauwolffia serpentine* Benth. E Kurz para comprovar a atividade de glicolisação desta planta *in vitro* (Schroeder et al., 1996). Schroeder et al. (1996) também isolaram piceina (**3e**), composto relacionado com a marcação de danos físicos nos tecidos celulares das plantas. Sircar et al. (2007) usaram o sistema de raízes transgénicas para a produção de ácido *p*-hidroxibenzóico (**3f**) em *Daucus carota* L. mostrando que a acumulação deste composto ocorre no citosol e na parede celular, sendo por isso um modelo promissor para o estudo biossintético deste compostos. Por outro lado, o ácido cafeoilquínico (**3g**) foi produzido em plântulas e *calli* de *Solidago graminifoli* L. e *Solidago virgaurea* L., plantas tradicionalmente usadas na Polónia pela maioria da população pelas suas caraterísticas medicinais que podem ser atribuídas à presença de ácidos fenólicos (Thiem et al., 2011). No mesmo estudo, um éster fenólico glicosilado, leiocoposídeo (**3h**) foi também isolado, demonstrando a atividade urológica deste compostos, e por isso com grande interesse de ser produzido em larga escala (Thiem et al., 2011).

Com menos significado numérico, mas com importância bioativa, a classe dos flavonoides, especialmente flavonas, são também alvos para a produção e isolamento em sistemas de cultura *in vitro*. Nishikaw et al. (1999) produziram um derivado de flavona (**3i**, 5,2'-di-hidroxi-6,7,8,3'-tetrametoxiflavona) em sistema de raízes transgénicas de *Scutellaria baicalensis* Georgi, tradicionalmente usadas pelas suas raízes para o tratamento da hepatite, tumores, diarreia e doenças inflamatórias. Shinde et al. (2010) também isolaram uma isoflavona numa cultura de calli de *Psoralea corylifolia* L. testando-a pela sua atividade

antioxidante, que foi maior sob condições de luz constante, sendo correlacionada com a maior presença de isoflavona. Por outro lado, Yang et al. (2001) isolaram uma isoflavona (**3j**) em calli de *Mirabilis jalapa* L. pela sua atividade antifúngica contra *Candida albicans*. Neste estudo, um segundo composto foi também isolado (desidro-rotenóide, **3k**) também com atividade antifúngica. Ziaratnia et al. (2009) isolaram um novo composto em cultura de calli de *Helichrysum aureonitens* L. Moench, clorofenol (**3l**), que foi testado pela sua atividade antitumoral e antituberculose. Os autores reconheceram a necessidade de estudos futuros para avaliar o seu potencial como molécula anticancerígena. Finalmente, Pinhatti et al. (2010) isolaram dois novos compostos fenólicos nas partes aéreas de *Hypericum ternum* A. St Hill, hiperosídeo e uliginosina (**3m**), observando que os níveis produzidos *in vitro* são significativamente mais elevados do que nas plantas silvestres, sendo necessária uma otimização do método para produzir estes compostos farmacologicamente pretendidos.

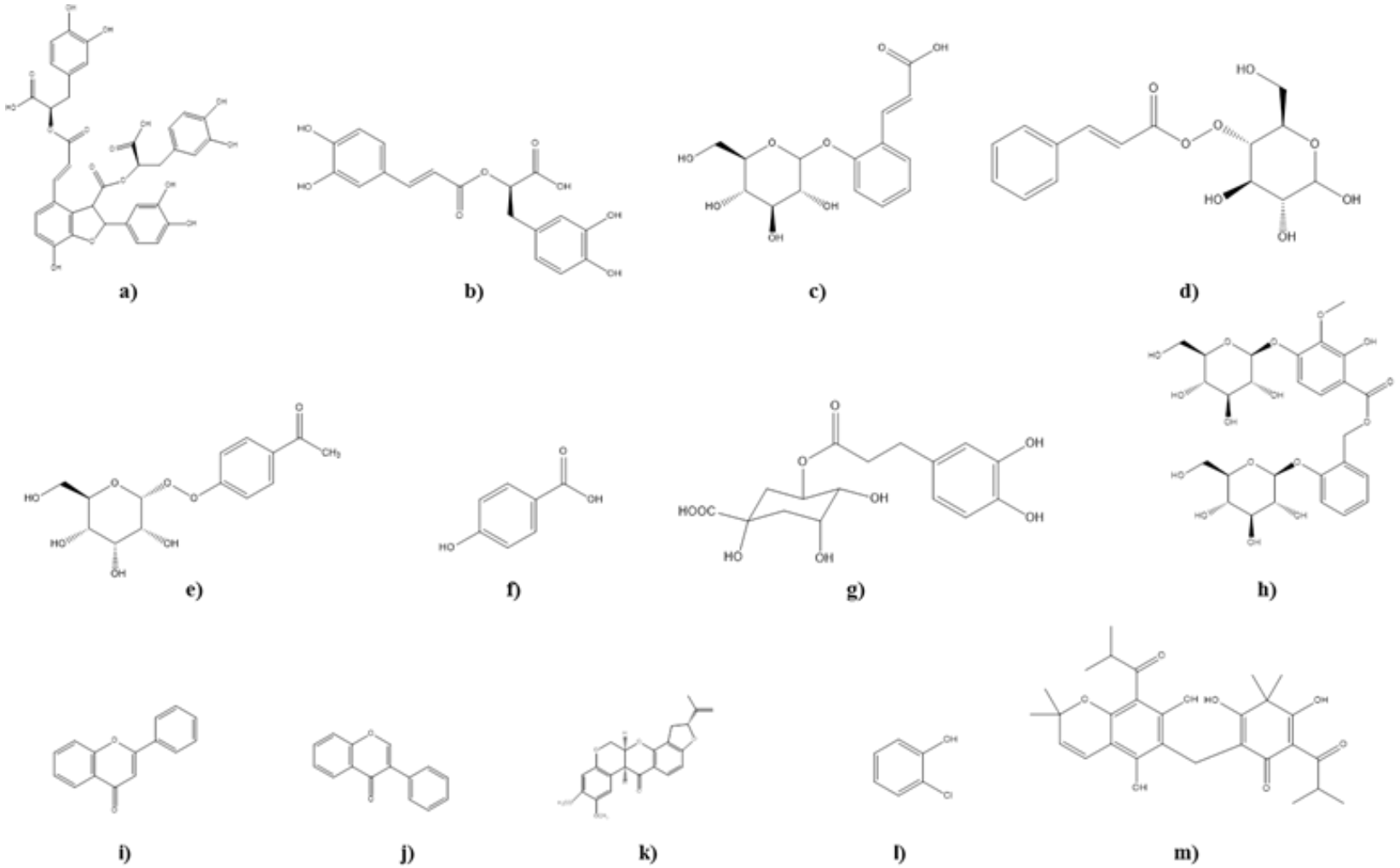


Figura 5. Exemplos de alguns compostos fenólicos individuais produzidos por técnicas de cultura *in vitro*: a) ácido litospémico B; b) ácido rosmarínico; c) ácido *o*-coumárico glicosilado; d) ácido cinâmico glicosilado; e) piceina; f) ácido *p*-hidroxibenzóico; g) ácido cafeoilquínico; h) leiocoposídeo; i) flavona; j) isoflavona; k) desidro-rotenóide; l) clorofenol; m) uliginosina (Dias et al., 2016).

2.1.3.2. Incremento na produção in vitro através do uso de elicitores

Apesar das plantas produzirem naturalmente compostos fenólicos quando colocadas *in vitro*, como descrito na secção anterior, existem muitas situações onde é necessário melhorar essa produção. Devido à breve fase estacionária que as plantas cultivadas *in vitro* apresentam, os metabolitos secundários são geralmente produzidos em baixas concentrações (inibição da ação enzimática, normalmente apresentada nas plantas mais maduras) (Michael & John, 1985). A elicitação é usada para aumentar a produção e acumulação de metabolitos secundários em sistemas de produção *in vitro*, acionando respostas morfológicas e fisiológicas por parte das plantas. Este estímulo ocorre em resposta ao stresse provocado por compostos de sinalização que ativam o mecanismo de resposta das plantas (Rea et al., 2011). A elicitação química é conseguida através do uso de fitorreguladores, moléculas de sinalização e pela adição de moléculas percursoras. A elicitação física envolve o uso de irradiação UV, pressão, campo elétrico e concentração de metais pesados. Os microorganismos, fungos e bactérias, podem funcionar como elicitores biológicos (Inga, et al, 2011; Baenas et al., 2014). Na **Tabela 3** estão descritos os principais grupos de elicitores usados para o incremento da produção de compostos fenólicos em sistemas de cultura de tecidos de plantas. A elicitação biológica é baseada, como dito anteriormente, na inoculação de bactérias e fungos que estimulam a via dos fenilpropanóides em resposta ao ataque microbiológico, melhorando a produção de fenólicos e em alguns casos atingindo maior produção de biomassa (Al-Amier et al., 1999; Verpoorte et al., 1999). Em termos de estirpes bacterianas, *Pseudomonas* sp. são as mais usadas, levando a uma maior produção de ácido rosmarínico em clones de *Lavandula angustifolia* Mill. (Al-Amier et al., 1999) e rebentos de *Rosmarinus officinalis* L. (Yang et al., 1997). Também aumentou a produção de compostos fenólicos em rebentos de *Thymus vulgaris* L. (Shetty et al., 1996). Nos três estudos foi observada também uma maior formação de rebentos, levando a uma maior produção de biomassa. Muitos dos estudos de elicitação, para além do objetivo de obter maior produção de compostos, são muitas vezes direcionados para a elucidação dos mecanismos de defesa da planta contra fungos. Alami et al. (1998) estudaram a produção de fitoalexinas de hidroxicumarinas, compostos fenólicos envolvidos na resistência das plantas, em *calli* de *Platanus acerifolia* Aiton elicitado com *Ceratocystis fimbriata* f. sp. Platani. Chegaram à conclusão que uma glicoproteína proveniente do fungo induzia a produção de mais 80% de cumarinas, excretadas para o meio de cultura. A produção de xantonas aumentou dez vezes mais em cultura de células de *Hypericum perfuratum* L. após elicitação com *Colletotrichum gloeosporioides* (Conceição et al., 2006). O mesmo foi verificado com os derivados do hidroxicinâmico em cultura de *calli* de *Phoenix dactylifera* elicitado com *Fusarium oxysporum* f. sp. albedinis (Daayf et al.,

2003). Estes dois estudos demonstraram que as plantas produzem níveis elevados de compostos fenólicos em cultura como mecanismo de defesa quando em presença de fungos. Em cultura de células de *Nicotina tabacum* L., após elicitação com *Phythophtora megasperma* f. sp. Glycinea, foi observado um aumento dos fenólicos ligados à parede celular (Ikemeyer & Barz, 1989). Hrazdina (2003) observaram uma produção diferencial de compostos fenólicos nas folhas e caules de cultura *in vitro* de *Malus domestica* Borkh cv Liberty e cv McIntos elicitadas com extrato de levedura e *Venturia inaequalis*. Num estudo conduzido por Vuković et al. (2013), a elicitação não foi feita com o contato direto do fungo com a planta, mas realizaram uma transformação genética usando *Agrobacterium rhizogenes* contendo o gene codificante para a proteína β-criptogeina (produzida por *Phytophthora cryptogea*) mimetizando o ataque de um patogénico, induzindo um mecanismo de defesa da planta que resultou numa maior acumulação de ácidos rosmarínico e cafeico.

A elicitação química pode ser obtida acionando uma resposta morfológica e fisiológica simplesmente adicionando compostos químicos ao meio de cultura que interferem com as vias biossintéticas que levam à produção de compostos fenólicos (Dong et al., 2010). De fato, muitos percursores do metabolismo secundário (via fenilpropanóide) são derivados do metabolismo primário, que no sentido de um equilíbrio entre crescimento e defesa da planta, vai direcionar para a produção dos metabolitos necessários (Lattanzio et al., 2009). Há inúmeros elicitores químicos: aminoácidos, compostos orgânicos e fitorreguladores. O aminoácido prolina é um dos mais usados como elicitor da produção de compostos fenólicos, tendo sido usado por Lattanzio et al. (2009) e Yang & Shetty (1998) em rebentos e *calli*, e também partes aéreas, respetivamente, de *Origanum vulgare* L. crescido *in vitro*. Em ambos os estudos a prolina estimulou a via das pentoses fosfato que está diretamente liga à via do chiquimato e dos fenilpropanóides, observando uma maior acumulação de compostos, como os ácidos rosmarínico, cafeico e litospérmico (Yang & Shetty, 1998; Lattanzio et al., 2009).

Tabela 3. Tipos de elicitação e respetivo grupo de elicitores usados em cultura *in vitro* para incremento da produção de compostos fenólicos (Dias et al., 2016).

Classe	Grupo	Elicitor	Origem	Referência
Elicitação biológica	Bactéria	*Pseudomonas mucidolens*	*Lavandula angustifolia* Mill. (rebentos)	Al-Amier et al., 1999
		Pseudomonas sp.	*Rosmarinus officinalis* L. (rebentos)	Yang et al. 1997
		Pseudomonas sp.	*Thymus vulgaris* L. (rebentos)	Shetty et al., 1996
	Fungo	*Ceratocystis fimbriata* f. sp. platani	*Platanus acerifolia* Aiton (calli)	Alami et al. 1998
		Colletotrichum gloeosporioides	*Hypericum perforatum* L. (células)	Conceição et al. 2006
		Fusarium oxysporum f. sp. albedinis	*Phoenix dactylifera* (calli)	Daayf et al., 2003
		Phythophtora megasperma f. sp. glycinea	*Nicotiana tabacum* L. (células)	Ikemeyer & Barz 1989
		Yeast extract and *Venturia inaequalis*	*Malus domestica* Borkh cv Liberty and cv McIntos (folhas e caules)	Hrazdina, 2003
		Indução genética	*Coleus blumei* Benth.(raízes transgénicas)	Vuković et al., 2013
Elicitação química	Aminoácidos	Hidrolisado de caseína e L-fenilalanina	*Ephedra alata* Decne. (calli)	Hegazi & El-Lamey 2011
		Prolina	*Origanum vulgare* L. ssp. Hirtum (rebentos e calli)	Lattanzio et al. 2009
		Prolina	*Origanum vulgare L.* (partes aéreas)	Yang & Shetty, 1998
	Condições de cultura	Densidade de inóculo e volume de aeração	*Eleutherococcus koreanum* Nakai (raízes)	Lee et al., 2011
	Compostos orgânicos	Ácido jasmónico e ácido salicílico	*Vitis vinifera* L. cv. Gamay Fréaux (calli e células)	Mewis et al. 2011
		Ácido salicílico	*Salvia miltiorrhiza* Bunge (calli)	Dong et al. 2010
		Glifosato	*Zea mays* L. (calli)	Ulanov et al., 2009
	Percursores	Fenilalanina	*Vitis vinifera* cv. Gamay Red (calli)	Krisa et al. 1999
		Ficioanina	*Capsicum frutescens* L. (calli)	Rao et al. 2006
		Ficioanina	*Daucus carota*L.(calli)	Rao et al. 2006
		Percursor	*Catharanthus roseus* L. (calli)	Shimoda et al., 2002
	Fitorreguladores	Citoquinas	*Merwilla plumbea* (Lindl.) Speta (partes aéreas e raízes)	Aremu et al., 2013
		Citoquinas	*Vitis vinifera* L. (calli)	Ozden & Karaaslan, 2011
		Fatores de transcrição	*Zea mays* L. (células)	Dias & Grotewold 2003
		Vários	*Brassica oleracea* L. var. costata (rebentos, raízes e calli)	Taveira et al. 2009
		Vários	*Genista tinctoria* L (calli)	Luczkiewicz et al., 2014
		Vários	*Hydrocotyle bonariensis* Lam. (calli)	Masoumian et al., 2011
Elicitação física	Compostos químicos	Cádmio	*Camellia sinensis* L. (calli)	Zagoskina et al. 2007
		Cobre	*Panax ginseng* sp. (raízes)	Ali et al. 2006
		Magnésio	*Vitis vinifera* cv. Gamay Red (células)	Sinilal et al. 2011
	Luz	Luz	*Eucalyptus camaldulensis* Dehn.(partes aéreas)	Arezki et al., 2011
		UV-A	*Phyllanthus tenellus* L. (olhas)	Victório et al. 2011
		UV-B	*Camellia sinensis L.*Georgian variety (calli)	Zagoskina et al. 2003
		UV-B	*Camellia sinensis* L. (calli)	Zagoskina et al. 2005
		UV-B	*Origanum vulgare* L.(rebentos)	Kwon et al., 2009
	Outros	Campo elétrico	*V. vinifera* L. cv. Gamay Fréaux (células)	Cai et al. 2011 B
Vários		Ácido ascórbico, carvão activado e fitorreguladores	*Strelitzia reginae* Banks (partes aéreas)	North et al., 2012

Ácido salicílico, sacarose, cuscuta	*Cayratia trifólia* L.(calli)	Arora et al. 2010
Compostos orgânicos	*Merwilla plumbea* (Lindl.) Speta (rebentos e raízes)	Baskaran et al., 2012
Compostos orgânicos	*Coleonema pulchellum* I.Williams (partes aéreas)	Baskaran et al., 2014
Estreptomicina, carvão ativado, ethepon e pressão hidrostática	*V. vinifera* L. cv. Gamay Fréaux (células)	Cai et al. 2011 A
Extrato de levedura e quitosano	*Curcuma mangga* Valeton & van Zijp (rebentos)	Abraham et al., 2011
Fitorreguladores,luz e sacarose	*Zingiber zerumbet* Smith (calli)	Stanly et al. 2011
Fusarium solani f.sp. Robiniae, jasmonato de metilo	*Nicotiana tabacum* L.(células)	Sharan at al. 1998
Sacarose e espermidina	*Rosa* sp. (L.) cv Paul's scarlet (células)	Muhitch & Fletcher 1985
Vários e fatores nutricionais	*Eryngium maritimum L.* (rebentos e raízes)	Kikowska et al., 2014

Hidrolisado de caseína e L-fenilalanina têm sido utilizados na indução da produção de fenólicos em calli de *Ephedra alata* Decne., conduzindo a uma maior acumulação de ácido clorogénico, rutina, quercetina e ácido cumárico (Hegazi & El-Lamey, 2011). Em termos de elicitores orgânicos, os ácidos salicílico e jasmónico são dois exemplos de compostos usados em cultura *in vitro* para induzir a produção de compostos fenólicos, sendo ambos moléculas de sinalização de diferentes vias biossintéticas. Enquanto o ácido jasmónico está envolvido na ativação da via de sinalização octadecanóide, o ácido salicílico induz a sinalização dos fenilpropanóides, no entanto, ambos respondem a ataques mecânicos e químicos contras as plantas (Mewis, 2011). O ácido salicílico provou estimular a ativação da enzima PAL (fenilalanina amônia-liase) em *calli* de *Salvia miltiorrhiza* Bunge levando à acumulação de ácido salvianólico B e ácido cafeico (Dong et al., 2010). Foi também comprovado que o mesmo aumenta a produção de biomassa e a concentração de antocianinas em cultura de *calli* e células de *Vitis vinifera* L. cv. Gamay Fréaux (Mewis, 2011). Por vezes, compostos que são normalmente usados como herbicidas podem também funcionar como elicitores em cultura *in vitro*, sendo um exemplo disso o composto glifosato usado para aumentar a produção de fenóis em cultura de calli de *Zea mays* L., causando uma maior acumulação de ácido chiquímico e quínico (Ulanov et al., 2009). Alguns elicitores orgânicos podem também funcionar de outra maneira, inibindo a produção de compostos fenólicos para prevenir, por exemplo, a oxidação das células (levando à sua morte) causada precisamente pela presença dos compostos fenólicos. Um exemplo é apresentado no trabalho desenvolvido por Jones & Saxena (2012), que usaram ácido 2-aminoindano-2-fosfónico para inibir a via fenilpropanóide em calli de *Acer saccharum* Marsh., *Artemisia annua* L. e *Ulmus Americana* L. A elicitação química envolve também o uso de moléculas percursoras que induzem a produção de derivados de fenólicos. Rao et al. (1996) utilizaram ficioanina em *calli* de *Capsicum frutescens* L. e *Daucus Carota* L. produzindo duas vezes mais capsaicinas e antocianinas do que nas culturas originais. No entanto, a maioria das investigações direciona-se para outro tipo de estudos, como a adição de percursor a *calli* de *Catharanthus roseus* L. para determinar a capacidade de glicosilação e hidroxilação da planta (Shimoda et al., 2003). Num outro estudo, utilizou-se o percursor de compostos antociânicos marcado com fenilalanina para estudar as vias de produção de antocianinas em *calli* de *Vitis vinifera* cv. Gamay Red (Krisa et al., 1999). A indução genética faz também parte da elicitação química, especialmente fatores de transcrição que permitem o controlo de determinadas proteínas envolvidas na biossíntese de compostos fenólicos. Dias & Grotewold (2003) induziram os genes R2R3 Myb contendo o fator de transcrição ZmMyb-IF35 em cultura de células de *Zea mays* L., tendo observado uma acumulação de ácidos ferúlico e clorogénico, que não estavam presentes nas amostras controlo.

Como foi dito anteriormente, os fitorreguladores revolucionaram a cultura de tecidos vegetais, principalmente devido ao fato do equilíbrio entre duas ou mais hormonas poder induzir o crescimento e desenvolvimento de diferentes órgãos e células nas plantas. No entanto, os fitorreguladores podem também induzir elicitação química e aumentar a produção de compostos fenólicos. As citoquinas foram usadas como elicitores em partes aéreas e raízes de *Merwilla plumbea* Lindl. Speta (Aremu et al., 2013) e em *calli* de *Vitis vinifera* L. (Ozden & Karaaslan, 2011) mostrando um aumento significativo de compostos, especialmente ácido vanílico em *M. plumbea*. Luczkiewicz et al. (2014), após testarem diferentes fitorreguladores em diferentes concentrações e agrupados de maneiras distintas, também observaram uma maior produção de isoflavonas em *calli* de *Genista tinctoria* L. elicitada com citoquinas.

Finalmente, a elicitação física, assim como a química, pode ser obtida com fatores abióticos que não têm origem biológica. Representa uma alternativa consistente para aplicações a larga escala, uma vez que permite aplicação contínua dos elicitores físicos sem contaminar os compostos bioativos e a cultura de planta (Rea et al., 2011). Algumas substâncias usadas neste tipo de elicitação são elementos químicos, como o cádmio, cobre e magnésio. O cádmio foi aplicado em cultura de *calli* de *Camellia sinensis* L. para estudar as mudanças metabólicas causadas por este metal pesado, observando uma mudança notória na composição de lenhina e flavanois nesta cultura (Zagoskina et al., 2007). O cobre foi usado para aumentar a produção de fenólicos em cultura de raízes de *Panax ginseng* sp., aumentando a produção de compostos fenólicos e flavonoides em 76% (Ali et al., 2006). Resultados semelhantes foram obtidos para a cultura de células de *Vitis vinifera* cv. Gamay Red na qual a aplicação de magnésio aumentou quatro vezes a acumulação de antocianinas (Sinilal et al., 2011). Diferentes comprimentos de onda de radiação têm também sido usados para a elicitação física. Uma vez que a luz é um dos fatores que mais stresse provoca na planta, os compostos fenólicos surgem como filtros UV ativos, protegendo a planta de possíveis danos. Os foto-receptores envolvidos no desenvolvimento dependente da luz das plantas incluem uma família de flavoproteínas (criptocromos) que causam uma variedade de respostas morfo-anatómicas, incluindo a produção de compostos fenólicos (Victório et al., 2011). Arezki et al. (2001) verificaram que uma simples mudança para um fotoperíodo de 16 horas aumentava o conteúdo de compostos fenólicos em partes aéreas de *Eucalyptus camaldulensis* Dehn. O comprimento de onda mais usado da luz UV é o B, no entanto Victório et al. (2011) verificaram que a luz UV-A aumentava o conteúdo de ácido elágico e derivados de elagitaninos mas, ao mesmo tempo, conduzia a uma redução no número de caules e rebentos de *Phyllanthus tenellus* L. Kwon et al. (2009) e Zagoskina et al. (2003) observaram um aumento no conteúdo fenólico em rebentos de *O. vulgare* e calli de *C. sinensis*, respetivamente, após tratamento com luz UV-B sem provocar danos nos

tecidos. Por outro lado, Zagoskina et al. (2005) verificaram que a concentração dos compostos fenólicos não era constante dependendo se a subcultura de *calli* de *C. sinensis* sofria elicitação com luz UV, o que leva a concluir que a produção de fenóis com elicitação usando luz é muito mais complexa. Pouco se sabe sobre o uso de campos elétricos em protocolos de cultura de células e tecidos; no entanto, tem sido comumente usada na indústria alimentar para descontaminação/eliminação de microorganismos. Mas uma vez que é um fator de stresse para as células vegetais, foi proposto o seu uso para elicitação da produção de metabolitos secundários. Cai et al. (2001b) estudaram os efeitos do campo elétrico combinado com fitorreguladores numa cultura de células de *Vitis vinifera* cv. Gamay Fréaux e obtiveram rendimentos mais elevados para a produção de antocianinas e compostos fenólicos, em comparação com as amostras controlo.

Por vezes, os investigadores têm necessidade de combinar vários procedimentos de elicitação para aumentar a eficiência do processo. Um dos exemplos mais comuns é a elicitação combinada entre diferentes fitorreguladores e fatores nutricionais do meio de cultura. Dois exemplos deste tipo de estudos são os realizados por Kikowska et al. (2014) em rebentos e raízes de *Eryngium maritimum* L. e por North et al. (2012) em partes aéreas de *Strelitzia reginae* Banks. O estudo realizado por Stanly et al (2011) em calli de Zingiber zerumbet Smith, demonstrou que a combinação de 2,4-D (Ácido 2,4-diclorofenoxiacético), cinetina, picloram, ANA (ácido naftalenoacético), sacarose e fotoperíodo aumentava a concentração de compostos antioxidantes. Sharan et al. (1998) estudou a produção de cumarinas (escopoletina e escopolina) em cultura de células de *Nicotina tabacum* L. elicitada com o fungo patogénico *Fusarium solani* f.sp. Robiniae e também jasmonato de metilo, tendo este último conduzido a uma maior acumulação de cumarinas. Baskaran et al. (2012) e Baskaran et al. (2014) estudaram o efeito de vários elicitores em rebentos e raízes de *Merwilla plumbea* (Lindl.) Speta e partes aéreas de *Coleonema pulchellum* I. William, respetivamente. Os elicitores usados foram diferentes fitorreguladores, aminoácidos e extrato de levedura. Em *M. plumbea* a produção de fenólicos foi 3 a 16 vezes maior do que nas culturas originais; a cultura de *C. pulchellum* demonstrou maior atividade antibacteriana com a combinação de elicitores. No estudo realizado por Abraham et al. (2011) em rebentos de *Curcuma manga* Valeton & van Zijp, doi observada uma maior concentração de antioxidantes combinando extrato de levedura e quitosano na elicitação. Cai et al. (2001a) combinaram estreptomicina, carvão ativado, etepon e pressão hidroestática para aumentar a produção de compostos em cultura de células de *Vitis vinifera* L. cv. Gamay Fréaux, observando que as concentrações de ácidos fenólicos eram mais elevadas que no controlo; a produção de antocianinas e biomassa não foi afetada.

Como o principal objetivo deste capítulo consiste numa revisão bibliográfica da produção de compostos fenólicos em cultura de células e tecidos vegetais com e sem

elicitação é importante saber que os próprios compostos fenólicos podem ser usados como elicitores. Através do conhecimento da via biossintética dos compostos fenilpropanóides, os investigadores chegaram à conclusão que a adição exógena de percursores ou intermediários de compostos fenólicos pode induzir ou aumentar os rendimentos de produção dos compostos em estudo (Palacio et al., 2011). Na **Tabela 4**, estão descritos exemplos de estudos onde os compostos fenólicos foram usados como elicitores.

Tabela 4. Compostos fenólicos usados como elicitores em estudos de cultura *in vitro* (Dias et al., 2016).

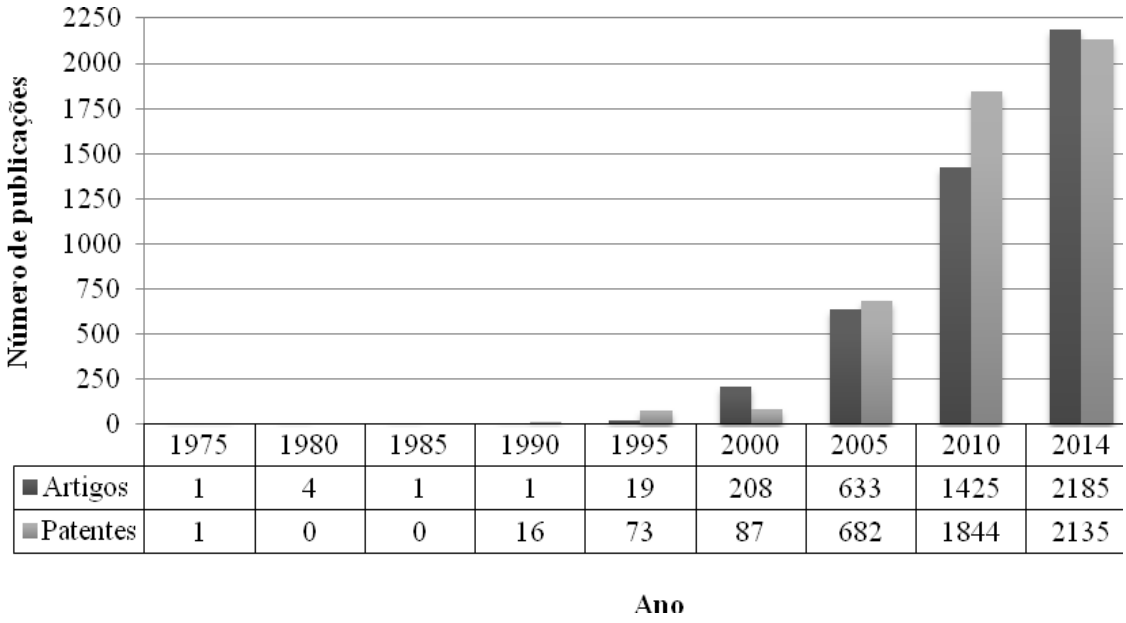
Elicitor	**Origem**	**Solvente de extração**	**Referência**
Ácido cafeico, floridizina e floroglucinol	*Feijoa sellowiana* Berg (embriões zigóticos)	Metanol	Reis et al. 2008
Ácido cinâmico, ácido ferúlico e ácido sinápico e L-fenilalanina	*Larrea divaricata* Cav. (calli)	Etanol	Palacio et al. 2011
Ácido clorogénico	*Hypericum perforatum* L. (células)	Água:Metanol (10:90, *v/v*)	Franklin & Dias 2011
Ácido gálico, ácido indolacético e cisteína	*Saccharum species* (rebentos)		Lorenzo et al., 2001
Compostos fenilpropanóides	*Saccharum officinarum* spp., cv.Badila. (meristemas)		Arencibia et al. 2008

Lorenzo et al. (2011) estudaram o efeito da adição do ácido gálico em rebentos de cana-de-açucar (espécie *Saccharum*) concluindo que, combinado com ácido indolacético e cisteína, ocorre uma maior excreção de compostos para o meio. Palacio et al. (2011) usaram os ácidos cinâmico, ferúlico e sinápico como elicitores em *calli* de *Larrea divaricata* Cav., enquanto Arencibia et al. (2008) estudaram a ação dos compostos fenólicos na indução de genes da via dos fenilpropanóides em partes aéreas de *Saccharum officinarum* spp., cv. Badila. No entanto, em ambos casos, são necessários estudos futuros para estabelecer a relação entre a elicitação e a produção dos compostos fenólicos. Outros estudos usaram os fenóis como promotores de crescimento e, consequentemente, obtenção de maior biomassa vegetal, nomeadamente os estudos realizados por Franklin & Dias (2011) e Reis et al. (2008) em cultura de células de *Hypericum perfuratum* L. e embriões zigóticos de *Feijoa sellowiana* Berg, respetivamente.

2.2. Microencapsulação de bioativos para aplicações alimentares

Atualmente a alimentação não serve apenas para satisfazer o desejo da fome, emergindo também como um meio para promover a saúde do consumidor. Neste contexto, a indústria alimentar tem-se centrado em evitar os malefícios associados aos aditivos sintéticos, promovendo o desenvolvimento de novos produtos alimentares contendo ingredientes com benefícios para a saúde. Assim, os produtos naturais bioativos são considerados substitutos viáveis e seguros para satisfazer uma procura mundial crescente (Mílner, 2010).

Os alimentos funcionais surgem na fronteira entre a nutrição e a saúde, providenciando a longo prazo um efeito fisiológico/saúde benéfico, para além das suas propriedades nutricionais (Mílner, 2010). O conceito de alimento funcional surgiu há 40 anos, no entanto o interesse por este tipo de produtos, seja por parte da indústria (através de patentes), ou em contexto académico (através de artigos de investigação e revisão), verificou-se apenas na segunda metade da década de 90, indicando uma tendência crescente (**Figura 6**).



	1975	1980	1985	1990	1995	2000	2005	2010	2014
Artigos	1	4	1	1	19	208	633	1425	2185
Patentes	1	0	0	16	73	87	682	1844	2135

Figura 6. Número de artigos de investigação e revisões, e patentes publicados entre o período compreendido entre 1970 e 2014 no tema dos alimentos funcionais (dados obtidos na web of science, Outubro de 2014; palavra-chave: “functional food”) (Dias et al., 2015).

O crescimento exponencial no nº de patentes e de artigos de investigação/revisão verificou-se a partir de 2005, o que foi acompanhado pela publicação do regulamento (EC) No 1924/2006 pelo Parlamento Europeu versando alegações nutricionais e de saúde nos alimentos, posteriormente complementada e finalizada em 2011 pela Autoridade Europeia para a Segurança dos Alimentos (“European Food Safety Authority” - EFSA) no que respeita

a alegações de efeitos benéficos para a saúde de certos ingredientes alimentares (Regulation (EC) No 1924/2006 European Parlament; Regulation (EC) No 1924/2006 EFSA). Nos Estados Unidos da América (EUA), o regulamento relativo aos alimentos funcionais está facilitada, sendo que a própria indústria alimentar atribui a definição do produto que vai ser colocado no mercado; esta é obrigada apenas a seguir o código de rotulagem e segurança implementado pela "Food and Drug Administration" (FDA) (FDA, 2004).

Hoje em dia, o consumidor está cada vez mais sensibilizado para as questões da saúde, coincidindo este comportamento com o aumento da incidência de doenças crónicas relacionadas com a idade, doenças neurodegenerativas, diabetes e cancro, isto é, doenças normalmente correlacionas com o estilo de vida e hábitos alimentares das sociedades atuais (Espín et al., 2007). Adicionalmente, com o aumento da esperança de vida e consequentes despesas com a saúde, as indústrias alimentares e farmacêuticas começam a considerar o mercado dos alimentos funcionais como de elevado potencial de crescimento. Atualmente, o Japão, EUA e a União Europeia (UE) são os mercados líderes em alimentos funcionais, representando 90% do mercado mundial deste tipo de produtos (Siró et al., 2008; Bigliardia & Galati, 2013). Em 2006, o mercado dos EUA e UE foi avaliado em 33 biliões e 15 biliões de USA$, respetivamente, com tendência para crescer. No contexto da UE, a Alemanha, França, Reino Unido e Holanda são os países mais representativos da comercialização de alimentos funcionais (Siró et al., 2008).

2.2.1 Microencapsulação de bioativos

2.2.1.1 Problemas relacionados com o uso de bioativos na forma livre

Apesar do conhecimento dos efeitos benéficos associados a matrizes naturais bioativas e aos seus compostos individuais isolados, como irá ser discutido nesta secção, estes extratos/compostos podem mostrar fragilidades que devem ser consideradas no seu uso direto ou quando incorporados em alimentos.

Os principais fatores limitantes no uso de bioativos em aplicações alimentares estão descritos na **Figura 7**

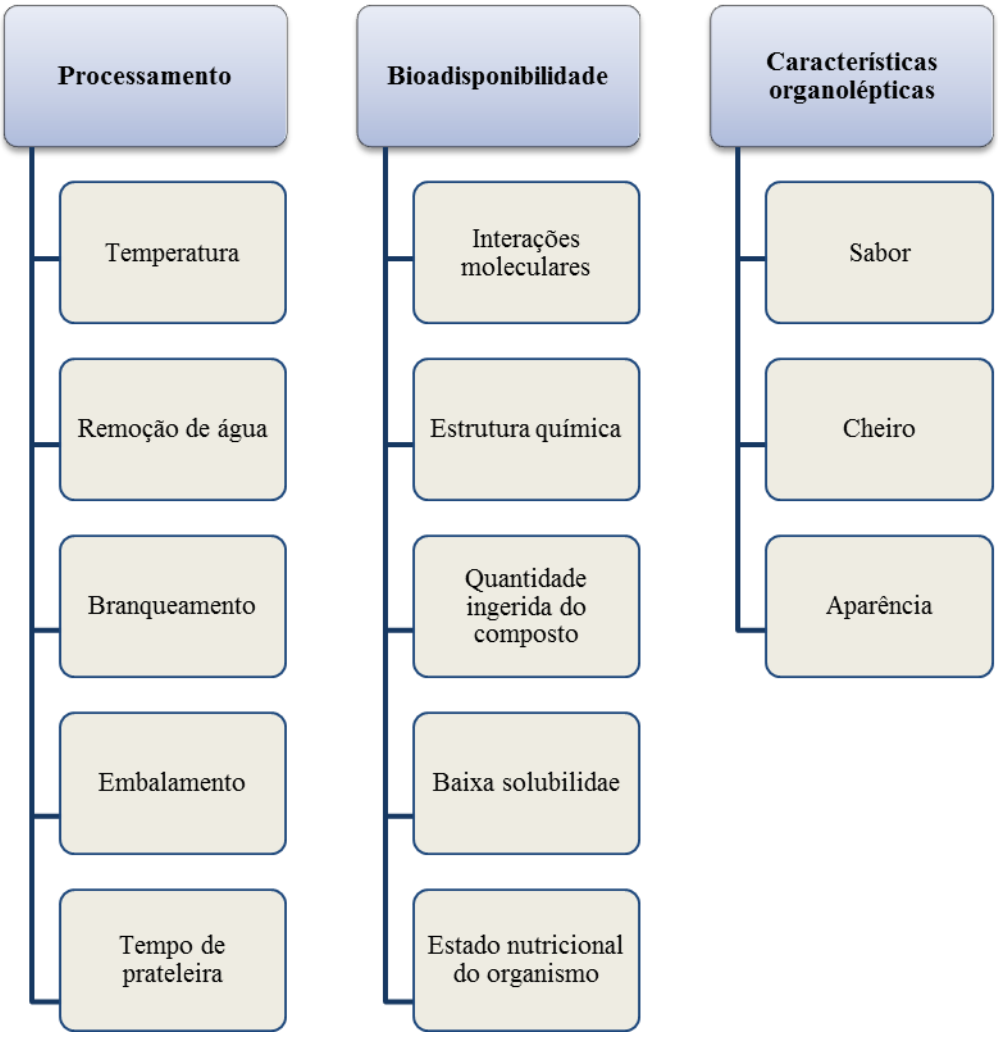


Figura 7. Fatores limitantes para o uso de bioativos na forma livre para fins alimentares (Dias et al., 2015).

Os ingredientes bioativos são geralmente propensos à degradação, durante o armazenamento e/ou processamento alimentar, pois muitos deles são física, química e/ou enzimaticamente instáveis, levando à sua degradação ou transformação com perda consequente de bioatividade. Em muitos casos, o mecanismo envolvido na degradação destas moléculas bioativas é complexo e ainda desconhecido (Espín et al., 2007; Joye et al.,

2014). Wu et al. (2010) reportaram a redução do conteúdo de antocianinas em amoras após seis meses embaladas e armazenadas como geleia, mesmo após tratamento de secagem. Vários tipos de cereais (trigo, cevada e aveia) foram também testados quanto ao seu conteúdo em compostos biologicamente ativos, nomeadamente em tocoferóis, compostos fenólicos e microelementos, tendo-se verificado que após processamento hidrotermal a concentração destas moléculas decresceu acentuadamente (Zielinski et al., 2001). Rawson et al. (2011) descreveram perdas de compostos bioativos acentuadas decorrentes do processamento de frutos exóticos, tais como manga, açaí, ananás e pitanga, relacionando-as com tratamentos térmicos, pasteurização e secagem, enlatamento e mesmo armazenamento. Todos estes processos afetam, de uma forma mais ou menos extensa, a estabilidade, as caraterísticas químicas e mesmo a atividade antioxidante de compostos como vitaminas e compostos fenólicos. Outro estudo onde se descrevem as modificações observadas em frutos e vegetais durante as etapas de processamento foi publicado por Nicoli et al. (1999). Este estudo refere o decréscimo da atividade antioxidante da matriz alimentar causada pela perda ou transformação dos compostos antioxidantes, mas também devida às interacções com outras moléculas da matriz.

As etapas de processamento de uma matriz alimentar dependem da acção de enzimas endógenas, da atividade da água, da presença de oxigénio e também da energia térmica/mecânica, podendo todos estes fatores influenciar a degradação/transformação de moléculas bioativas levando à perda das suas características. No entanto, nem todos os compostos são igualmente afetados; os compostos fenólicos e as vitaminas (ex. vitamina C e E) são mais sensíveis ao branqueamento e aos tratamentos de congelação a longo prazo, comparativamente aos minerais ou fibras alimentares (Puupponen-Pimiä et al., 2003).

Além do processamento, a perecibilidade dos alimentos é também uma limitação à ingestão de compostos bioativos na forma livre; o tempo de prateleira determina se um determinado alimento mantém as suas propriedades e caraterísticas bioativas. Por exemplo, os cogumelos comestíveis têm um tempo de prateleira muito curto e as mudanças após colheita, nomeadamente o acastanhamento, a transformação do chapéu, a alteração de textura e a perda de massa, levam ao decréscimo dos seus componentes bioativos (Fernandes et al., 2012a).

A quantidade ingerida do composto bioativo, a sua estrutura e composição química, a interação com outras moléculas, mas também o próprio organismo (massa da mucosa, comportamento gastrointestinal e interações com proteínas) vão influenciar a estabilidade e funcionalidade deste no organismo humano e, consequentemente, a sua biodisponibilidade (Holst & Williamson, 2008; Leong & Oey, 2012). Por exemplo, os compostos fenólicos apresentam baixa biodisponibilidade devido à sua baixa solubilidade e estabilidade, em particular os compostos de massa molecular elevada. Além disso, não há estudos sobre a

existência de recetores específicos para este tipo de compostos na superfície das células epiteliais no intestino delgado e, por isso, o mecanismo de transporte é feito por difusão ativa, diminuindo a sua permeabilidade (Li et al., 2015). Já as antocianinas, são muito sensíveis às mudanças de pH e temperatura do meio (Fernandes et al., 2014). Relativamente à classe dos carotenóides, a natureza da matriz alimentar, o tamanho das partículas, o método de processamento, mas também a interação com outros constituintes do alimento, vai afetar a sua biodisponibilidade; os constituintes da fibra, por exemplo, diminuem a absorção dos carotenóides. O estado nutricional do próprio organismo vai influenciar a absorção destas moléculas (p. ex. a deficiência proteica afeta a sua biodisponibilidade) (Rodriguez-Amaya, 2010; Fernández-García et al., 2012). Adicionalmente, a interação dos elementos minerais com outras moléculas pode diminuir a sua biodisponibilidade, tal é o caso do cálcio onde os compostos como os oxalatos, taninos e fibras dietéticas decrescem a absorção por precipitação dos compostos (Amalraj & Pius, 2015). O ambiente gastrointestinal e o transporte epitelial podem também diminuir a biodisponibilidade dos extratos naturais, tal como foi descrito por Vermaak et al. (2010) que investigou a atividade biológica do chá verde e extratos de sálvia simulando as condições gastrointestinais; os autores observaram uma diminuição acentuada na sua atividade antimicrobiana.

Os compostos lipofílicos têm também baixa solubilidade, o que restringe a sua incorporação em muitas matrizes alimentares, maioritariamente hidrofílicas. O peso molecular, a funcionalidade e a polaridade influenciam a solubilidade, estado físico, estabilidade química e biodisponibilidade (McClements et al., 2007; Joye et al., 2014). É muito difícil avaliar a biodisponibilidade deste tipo de compostos, após metabolizados entram no sistema circulatório onde podem ser armazenados, utilizados ou excretados. A sua biodisponibilidade depende da concentração, do tempo de armazenamento num dado tecido, ou da sua ação biológica (McClements & Li, 2007). Por exemplo, a biodisponibilidade do licopeno, um composto carotenóide altamente lipofílico, é extremamente influenciada pela absorção linfática intestinal. Faisal et al. (2013) aplicaram, *in vivo,* um modelo para aumentar a solubilidade usando excipientes lipídicos digestíveis. Um estudo semelhante foi realizado por Balakrishnan et al. (2010) para aumentar a solubilidade da Coenzima Q_{10}, praticamente insolúvel em meio aquoso, usando óleo e compostos surfactantes, para administração oral.

Outro fator alvo de investigação para o desenho de novos sistemas de libertação para a área alimentar é o comportamento organoléptico de alguns compostos/extratos bioativos. Estes podem apresentar sabores, aromas e mesmo texturas desagradáveis. Este é um ponto crucial na indústria alimentar aquando do desenvolvimento de novos produtos; o consumidor não só dá importância ao preço, mas também, e principalmente, ao sabor,

cheiro e aparência. Assim, os consumidores vão escolher, mesmo com propriedades bioativas inferiores, um produto não funcional equivalente (Bech-Larsen & Scholderer, 2007; Leong & Oey, 2012). De facto, muitas pessoas evitam o consumo de frutos e vegetais, que devido à presença de certos compostos fenólicos, terpenos e glucosinolatos, apresentam sabores amargos ou adstringentes, o que os torna pouco apelativos (Drewnowski & Gomez-Carneros, 2000).

Para ultrapassar os problemas relacionados com o uso direto de extratos/compostos bioativos, as técnicas de microencapsulação apresentam um elevado potencial de utilização na indústria alimentar, nomeadamente podem ajudar a conferir propriedades funcionais ou para a proteger os bioativos. Assim, o principal objetivo deste capítulo é evidenciar o uso das técnicas de microencapsulação na área alimentar, assim como discutir as vantagens associadas à microencapsulação dos compostos/extratos bioativos. Com base na literatura, serão enumerados vários extratos e compostos alvo de microencapsulação seguindo diferentes técnicas e formulações, assim como o seu potencial para o desenvolvimento de alimentos funcionais. Será dado particular enfase aos exemplos que abordam o desenvolvimento de uma aplicação final (incorporação em matrizes alimentares).

2.2.1. Resumo das técnicas e materiais para microencapsulação

2.2.1.1. Vantagens do uso de bioativos microencapsulados

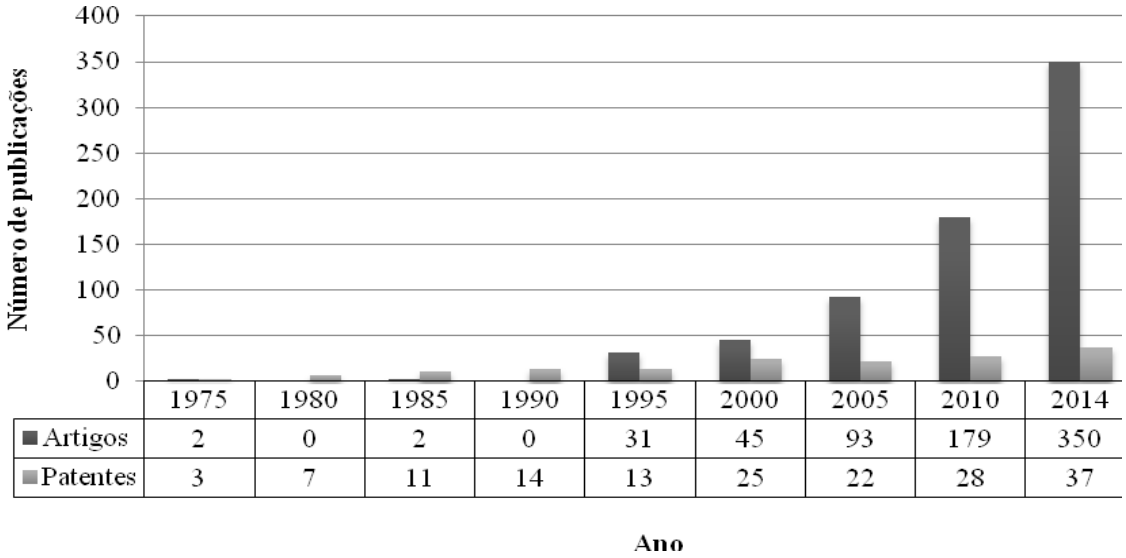
A microencapsulação pode fornecer uma ferramenta apta a proteger os extratos e compostos naturais da ação biótica, abiótica e de fatores biológicos. Emerge como uma metodologia viável para utilização na indústria alimentar, mas também no campo da nutrição e saúde, onde a estabilidade, eficácia e biodisponibilidade destes extratos é necessária. Como descrito anteriormente, existem muitos fatores que afetam a estabilidade do bioativo na sua forma livre (**Figura 7**), no entanto com a tecnologia de microencapsulação é possível protege-los de fatores ambientais como a luz, humidade, calor e oxigénio. Adicionalmente, as características organolépticas de muitos produtos alimentares podem ser mascaradas, mas, mais importante, as características funcionais/biológicas podem ser mantidas, mesmo após ingestão e/ou conseguir uma libertação controlada num alvo específico. O sucesso de um sistema de libertação baseado na microencapsulação pode ser medido pelo comportamento do bioativo durante o processamento e armazenamento do alimento e após a sua ingestão (Joye et al., 2014). Do ponto de vista prático, as técnicas de microencapsulação protegem o material do núcleo do ambiente externo; aumentam o tempo de prateleira do produto, dado que reduzem as transferências entre o núcleo e o meio circundante, e protegem as moléculas da reação com os outros constituintes do alimento

(Fang & Bhandari, 2010). Pode também promover o aumento da solubilidade e a capacidade de dispersão dos bioativos (Kuang et al., 2010).

Dependendo da tecnologia aplicada e do bioativo encapsulado, a resposta do sistema de libertação será diferente; para cada composto há características específicas que devem ser consideradas no desenho de um novo processo de microencapsulação. Por exemplo, os compostos fenólicos são poderosos antioxidantes, no entanto apresentam problemas de biodisponibilidade após ingestão derivados de transformações como metilações, glucorunações e sulfatações (Heleno et al., 2015). Assim, os sistemas de administração baseados em nano e micropartículas aparecem como uma solução para ultrapassar estes problemas, promovendo o aumento da absorção fitoquímica dos compostos fenólicos em células epiteliais (Wang et al., 2014; Li et al., 2015). Em particular, Davidov-Pardo & McClements (2014) demonstraram que a biodisponibilidade do resveratrol aumentou após microencapsulação. Os óleos essenciais apresentam também problemas dado as suas características organolépticas, a grande maioria tem um sabor e cheiro desagradável, baixa solubilidade e são altamente voláteis. Todas estas limitações podem ser ultrapassadas usando técnicas de microencapsulação que aumentam a eficácia das suas funções biológicas e diminuem o impacto sensorial nos produtos alimentares (Nazzaro et al., 2012).

2.2.1.2. Técnicas de microencapsulação

O conceito de microencapsulação foi primeiramente desenvolvido no setor da indústria farmacêutica, visando controlar e/ou modificar a libertação de medicamentos. Hoje em dia, representa ainda o maior setor de aplicação da microencapsulação (68%), enquanto a área alimentar representa apenas 13% (Martins et al., 2014a). O número de publicações científicas e patentes relativas à microencapsulação para fins alimentares (**Figura 8**) é indicativo do interesse crescente por esta técnica, nomeadamente no que respeita à incorporação de extratos e compostos bioativos. No entanto, a inexistência de regulamentação para novos ingredientes alimentares, incluindo para aqueles baseados em nano e microtecnologias, é ainda escassa. Nos EUA, a FDA está a desenvolver um programa de identificação de nanomateriais para ultrapassar a escassez de informação existente, e também para avaliar a segurança alimentar destes novos ingredientes (Kwak, 2014).



	1975	1980	1985	1990	1995	2000	2005	2010	2014
Artigos	2	0	2	0	31	45	93	179	350
Patentes	3	7	11	14	13	25	22	28	37

Figura 8. Número de artigos de investigação e revisões, e patentes publicados entre o período compreendido entre 1970 e 2014 relativamente à microencapsulação para fins alimentares (dados obtidos no web of science, Outubro de 2014; palavras-chave: "microencapsulation" e "food") (Dias et al., 2015).

A introdução de tecnologias de microencapsulação na indústria alimentar permite a incorporação de diversos aditivos em alimentos, mas também a melhoria das suas propriedades funcionais e de saúde (Kuang et al., 2010; Nedovic et al., 2011). Na biotecnologia e ciência alimentar, a incorporação de ingredientes naturais visa estabilizar, proteger e preservar os bioativos dentro de um núcleo, rodeado por um filme (cápsula), ou disperso numa matriz, fabricada de um material selecionado de forma adequada para o sistema de libertação pretendido (Nazzaro et al., 2012). Atualmente é possível encontrar algumas revisões sobre microencapsulação de compostos e extratos bioativos para aplicação alimentar (Schrooyen et al., 2001; Champagne & Fustier, 2004; Gouin, 2004; Fang & Bhandari, 2010; Kuang et al., 2010; Nedovic et al., 2011; Nazarro et al., 2012), no entanto, estas exploram maioritariamente as técnicas de microencapsulação disponíveis, e muito pouco o desenvolvimento de aplicações finais.

A Figura 9 mostra a cadeia sequencial lógica desde a escolha dos bioativos, materiais e processos de microencapsulação, até ao desenvolvimento da aplicação final, evidenciando os pontos críticos envolvidos em cada etapa.

As microcápsulas são partículas com diâmetros compreendidos ente 1 e 1000 micrómetros (μm). A morfologia principal pode ser dividida em dois tipos: (1) tipo "cápsula", onde o núcleo, contendo o bioativo e por vezes um transportador (composto que facilita a libertação), é protegido por uma membrana; (2) tipo "matriz", onde o bioativo está disperso no próprio material da matriz. Os materiais de encapsulação, o processo de produção, a morfologia e a aplicação final constituem os fatores mais importantes a ter em consideração quando se desenvolve um novo produto baseado num sistema de libertação. Quando se

seleciona a técnica de microencapsulação deve-se também ter em consideração a estabilidade e as propriedades funcionais do bioativo. Adicionalmente, para se obterem eficiências de encapsulação elevadas, assegurar a reprodutibilidade e a obtenção de um perfil de libertação adequado é necessário ultrapassar algumas restrições do processo como a agregação e a adesão das microsferas (Kuang et al., 2010).

Os métodos de encapsulação e os materiais mais usados para fins alimentares estão descritos na Tabela 5 e na Tabela 6, respectivamente. A divisão em categorias, tal como apresentada na Tabela 5, revestiu-se de alguma dificuldade dado que o processo de microencapsulação pode ser categorizado de acordo com o mecanismo de formação, o método de consolidação das micropartículas, ou refletir o equipamento específico usado. A distinção entre as categorias descritas nem sempre é clara nos artigos publicados. Assim, neste trabalho, foi feito um esforço para definir as categorias de acordo com o processo de formação da microcápsula, propondo-se o seguinte conjunto de categorias gerais: coacervação, processos baseados na tecnologia de extrusão, processos baseados na tecnologia de spray, processos baseados na preparação de emulsões, lipossomas, processos baseados na utilização de fluídos supercríticos, processos baseados na tecnologia de ultra-sons e outros.

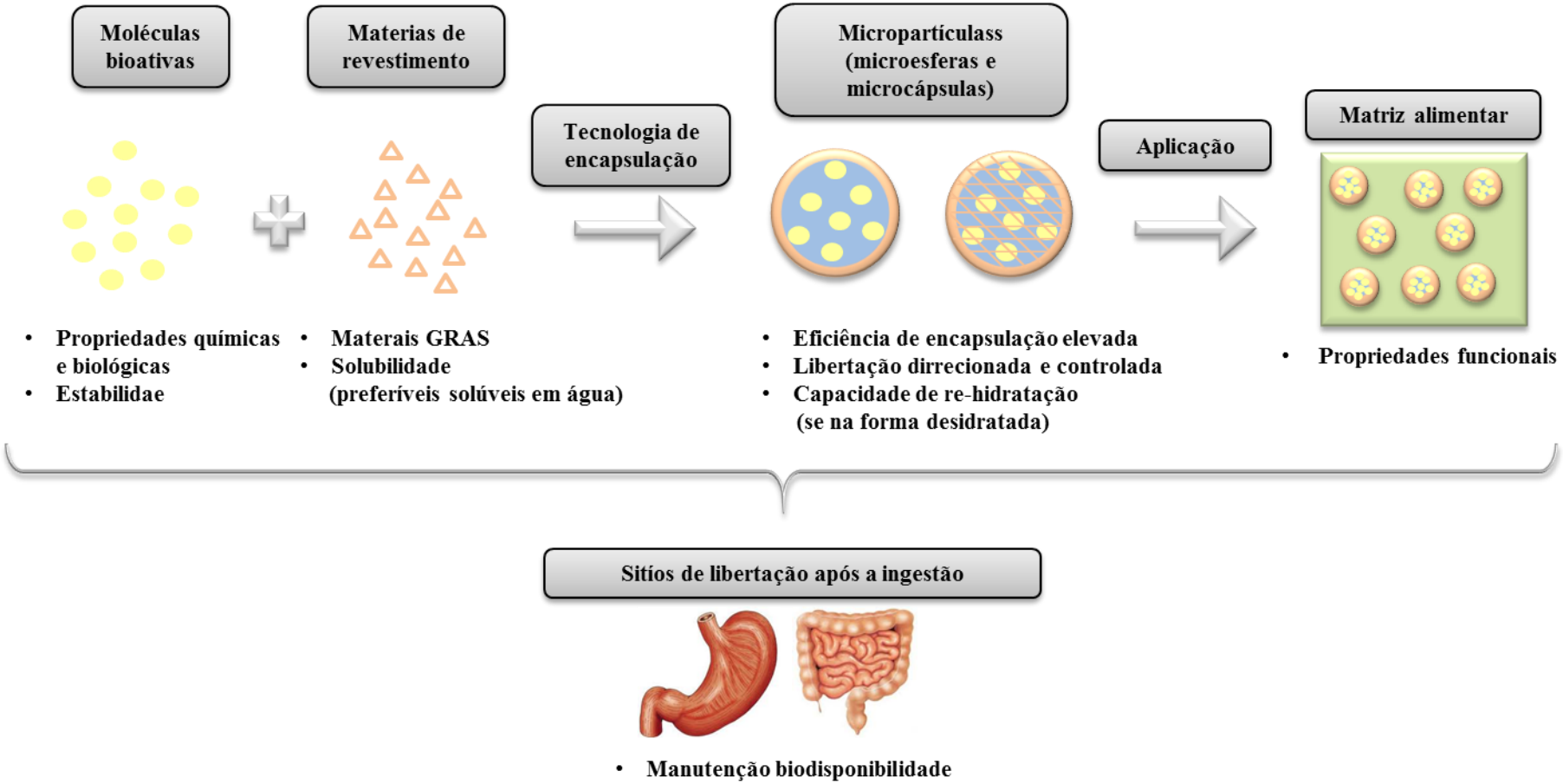


Figura 9. Esquematização do processo para o desenvolvimento de protocolos de microencapsulação (GRAS-"generally recognized as safe") (Dias et al., 2015).

Tabela 5. Metodologias de encapsulação mais usadas para fins alimentares e exemplos correspondentes (Dias et al., 2015).

Categoria do método	Exemplos	Referência
Coacervação	Coacervação complexa	Qv et al, 2011; Xu et al., 2014; Deladino et al., 2008; Chandy et al., 1998; Belščak-Cvitanović et al., 2011; Hui et al., 2013; Naik et al., 2014; Liang et al., 2011; Gibis et al., 2014; Madrigal-Carballo et al., 2010;
	Coacervação simples	Che net al., 2013; Averina & Alléman, 2013; Ostertag et al., 2012; Frank et al., 2012; Pan et al., 2014; Coimbra et al., 2011; Wu et al., 2008;
Processos baseados na extrusão	Extrusão electrostática	Belščak-Cvitanović et al., 2011; Barbosa-Pereira et al., 2014
	Co-extrusão	Chan et al., 2010; Piazza & Roversi, 2011
Processos baseados em spray	Secagem por spray	Ersus & Yurdagel, 2007; Nayak & Rastogi, 2010; Osorio et al., 2012; Berg et al., 2012; Tonon et al., 2010; Santa-Maria et al., 2012; Medina-Torres et al., 2013; Robert et al, 2012; Souza et al., 2013; Sansone et al., 2011a; Bakowska-Barczaka & Kolodziejczykb, 2011; Çam et al., 2014; Gallegos-Infante et al., 2013; Pang et al, 2014; Saénz et al., 2009; Sun-Waterhouse et al., 2013; Guadarrama-Lezama et al., 2012; Ahmed et al., 2010; Parthasarathi et al., 2013; Sansone et al., 2011b; Fernandes et al., 2012b; Bule et al., 2010; Silva et al., 2013: Baranauskiene et al., 2006; Adamiec et al., 2012; Costa et al., 2013; Garcia et al., 2012; Romo-Hualde et al., 2012; Aissa et al., 2012; Krishnaiah et al., 2012; Chiou & Langrish, 2007; Cortés-Rojas et al., 2014a; Igual et al., 2014; Langrish & Premarajah, 2013; Cortés-Rojas et al., 2014b; Ezhilarasi et al., 2013a; Gallardo et al., 2013; Ng et al., 2013; Pillai et al., 2012; Robert et al., 2010; Rocha-Guzmán et al., 2010; Rubilar et al., 2012; Sansone et al., 2014; Shaw et al., 2007; Souza et al., 2014;Bagheri et al., 2014; Chen et al., 2013; Gharsallaoui et al., 2012; Park et al., 2014
	Eletrospray	Pérez-Masiá et al., 2015
	Spray-coagulação*	Wichchukit et al., 2013;Deladino et al., 2008; Betancur-Ancona et al., 2011; Martins et al., 2014b; Chandy et al., 1998; Liang et al., 2011; Santos et al., 2013
	Spray-liofilização	Jung et al., 2013; Laine et al., 2008; Sanchez et al., 2011; Spada et al., 2012a; Spada et al., 2012b; Ezhilarasi et al., 2013b; Naik et al., 2014
Processos baseados na preparação de emulsões		Averina & Allémann, 2013; Chen & Subirade, 2006; Haidong et al., 2011; Augustin et al., 2011; Gupta & Ghosh, 2014; Malik et al., 2014; Ostertag et al., 2012; Stratulat et al., 2014; Vidal et al., 2012; Seok et al., 2003; Betz et al., 2012; Frank et al., 2012; Pan et al., 2014; Hui et al., 2013; Betz & Kulozika, 2011
Lipossomas	Lipossomas e niossomas	Barras et al., 2009; Coimbra et al., 2011; Gibis et al., 2014; Hasan et al., 2014; Madrigal-Carballo et al., 2010; Rasti et al., 2012; Tavano et al., 2014
Processos baseados em fluídos supercríticos	Processo do antisolvente	Sosa et al., 2011; Visentin et al., 2012
	Extração rápida numa solução supercrítica	Santos et al., 2013
	Impregnação em fluido supercrítico	Almeida et al., 2013

Processos baseados em ultra-sons	Sonificação	Kalogeropoulos et al., 2009; Cilek et al., 2012
	Ultra-sons	Mantegna et al., 2012
Outros	Co-cristalização	López-Córdoba et al., 2014; Sardar et al., 2013
	Impressão núcleo-parede	Blanco-Pascual et al., 2014
	Nanoprecipitação	Averina & Allémann, 2013
	Leito fluidizado	Li et al., 2007
	Inclusão	Ma et al., 2011; Zhao et al., 2010
	Liofilização	Rosa et al., 2013; Rutz et al., 2013
	Microondas	Abbasi et al., 2009
	Inclusão molecular	Kalogeropoulos et al., 2010
	Nanoprecipitação	Wu et al., 2008
	Método de separação de fases	Zheng et al., 2011
	Superfície de resposta	Lee et al., 2013
	Evaporação do solvente	Kumari et al, 2010; Prasertmanakit et al., 2009
	Separação por suspensão rotacional	Akhtar et al., 2014

* Coagulação por gelificação interna ou externa

Tabela 6. Principais materiais utilizados para a encapsulação de extratos bioativos e compostos para fins alimentares (com base em Kuang et al. 2010) (Dias et al., 2015)

Categoria	Material para encapsulação	Referência
Polímeros solúveis em água	Hidratos de carbono e seus derivados (ex.: alginato, gomas, quitosano, amilose, k-carragenina e pectina), proteínas e seus derivados (p. ex.: proteínas do soro de leite, leite e soja), polímeros sintéticos (p. ex.: polietileno glicol) e outros (p. ex.: etil celulose e extrato de mucilagem de *Opuntia ficus* Indica)	Chan et al., 2010; Silva et al., 2013; Chen & Subirade, 2006; Wichchukit et al., 2013; Deladino et al., 2008; Qv et al, 2011; Pérez-Masiá et al., 2015; Belščak-Cvitanović et al., 2011; Averina & Allémann, 2013; Gupta & Ghosh, 2014; Malik et al., 2014; Betz & Kulozika, 2011; Hui et al., 2013; Piazza & Roversi, 2011; Li et al., 2007; Ma et al., 2011; Liang et al., 2011; Madrigal-Carballo et al., 2010; Rosa et al., 2013; Rutz et al., 2013; Bagheri et al., 2014; Zheng et al., 2011; Santos et al., 2013; Santos et al., 2013; Lee et al., 2013; Prasertmanakit et al., 2009; Tonon et al., 2010; Santa-Maria et al., 2012; Medina-Torres et al., 2013; Robert et al, 2012; Souza et al., 2013; Sansone et al., 2011a; Bakowska-Barczaka & Kolodziejczykb, 2011; Gallegos-Infante et al., 2013; Pang et al, 2014; Sun-Waterhouse et al., 2013; Guadarrama-Lezama et al., 2012; Parthasarathi et al., 2013; Fernandes et al., 2012b; Bule et al., 2010; Baranauskiene et al., 2006; Adamiec et al., 2012; Costa et al., 2013; Garcia et al., 2012; Romo-Hualde et al., 2012; Aissa et al., 2012; Krishnaiah et al., 2012; Chiou & Langrish, 2007; Cortés-Rojas et al., 2014a; Igual et al., 2014; Langrish & Premarajah, 2013; Visentin et al., 2012; Cilek et al., 2012; Vidal et al., 2012; Abbasi et al., 2009; Martins et al., 2014b; Betancur-Ancona et al., 2011; Betz et al., 2012; Chandy et al., 1998; Chen et al., 2013; Ezhilarasi et al., 2013a; Ezhilarasi et al., 2013b; Gallardo et al., 2013; Naik et al., 2014; Ng et al., 2013; Pillai et al., 2012; Robert et al., 2010; Rubilar et al., 2012; Sansone et al., 2014; Shaw et al., 2007; Xu et al., 2014; Berg et al., 2012; Souza et al., 2013; Sansone et al., 2011b; Frank et al., 2012
Não polímeros solúveis em água	Hidratos de carbono e seus derivados (p. ex: ciclodextrinas, maltodextrina, inulina e lactose), polímeros sintéticos (p.ex.:PEG2000-DSPE, álcool polivinílico e emulsionantes polímeros lipofílicos HLP altos e baixos) e outros (Tween, tampão, soluções alcoólicas e ácido ascórbico)	Kalogeropoulos et al., 2010; Silva et al., 2013; Haidong et al., 2011; Jung et al., 2013; Laine et al., 2008; Sanchez et al., 2011; Zhao et al., 2010; Rosa et al., 2013; Lee et al., 2013; Ersus & Yurdagel, 2007; Nayak & Rastogi, 2010; Osorio et al., 2012; Berg et al., 2012; Tonon et al., 2010; Bakowska-Barczaka & Kolodziejczykb, 2011; Çam et al., 2014; Gallegos-Infante et al., 2013; Pang et al, 2014; Saénz et al., 2009; Guadarrama-Lezama et al., 2012; Ahmed et al., 2010; Sansone et al., 2011b; Fernandes et al., 2012b; Bule et al., 2010; Costa et al., 2013; Krishnaiah et al., 2012; Igual et al., 2014; Cilek et al., 2012; Mantegna et al., 2012; Ezhilarasi et al., 2013a; Ezhilarasi et al., 2013b; Gallardo et al., 2013; Kalogeropoulos et al., 2009; Ng et al., 2013; Robert et al., 2010; Rubilar et al., 2012; Souza et al., 2014; Qv et al, 2011; Averina & Allémann, 2013; Augustin et al., 2011; Malik et al., 2014; Ostertag et al., 2012; Ostertag et al., 2012; Li et al., 2007; Spada et al., 2012b; Coimbra et al., 2011; Tavano et al., 2014; Wu et al., 2008; Santos et al., 2013; Akhtar et al., 2014; Berg et al., 2012; Ahmed et al., 2010; Baranauskiene et al., 2006; Cortés-Rojas et al., 2014a; Rocha-Guzmán et al., 2010
Polímeros insolúveis em água	Hidratos de carbono e seus derivados (p. ex: amido), proteínas e seus derivados (p. ex: caseína), polímeros sintéticos (p. ex: polietileno de baixa densidade, poli (ε-caprolactona) e poli-D, L-ácido lactico (PLA) e outros (ex. vaselina líquida)	Kumari et al, 2010; Pérez-Masiá et al., 2015; Augustin et al., 2011; Park et al., 2014; Park et al., 2014; Stratulat et al., 2014; Pan et al., 2014; Barbosa-Pereira et al., 2014; Spada et al., 2012a; Spada et al., 2012b; Ma et al., 2011; Wu et al., 2008; Sosa et al., 2011; Tonon et al., 2010; Robert et al, 2012; Gharsallaoui et al., 2012; Bule et al., 2010; Costa et al., 2013; Almeida et al., 2013; Vidal et al., 2012; Abbasi et al., 2009; Frank et al., 2012; Rocha-Guzmán et al., 2010
Não polímeros insolúveis em água	Hidratos de carbono e seus derivados e outros (p. ex.: lecitina, CO_2 supercrítico CO2, ácido esteárico e cera)	López-Córdoba et al., 2014; Sardar et al., 2013; Malik et al., 2014; Barras et al., 2009; Coimbra et al., 2011; Gibis et al., 2014; Hasan et al., 2014; Madrigal-Carballo et al., 2010; Rasti et al., 2012; Lee et al., 2013; Almeida et al., 2013; Seok et al., 2003; Blanco-Pascual et al., 2014; Cortés-Rojas et al., 2014b; Shaw et al., 2007

Os processos baseados em *spray* são sem dúvida os métodos mais comuns, sendo divididos em secagem por spray, spray-coagulação (de acordo com o processo de gelificação interna ou externa) e spray-liofilização. A secagem por spray, o processo de microencapsulação mais antigo usado pela indústria alimentar, é uma técnica simples e de aplicação direta. Pode ser descrita como sendo um processo flexível, permitindo a produção em modo contínuo, tornando-se por isso um processo de baixo custo e, consequentemente, um dos mais económicos entre os vários métodos de encapsulação. Pode ser facilmente industrializável em termos de equipamento e materiais, tendo um baixo custo, comparativamente a outras técnicas disponíveis (Gharsallaoui et al., 2007). Os materiais de parede mais usados com esta técnica são os hidratos de carbono, o que pode limitar a encapsulação de alguns bioativos (Gouin, 2004). Originam microcápsulas de qualidade elevada, com um tamanho inferior a 40 μm, a partir da atomização de uma solução líquida ou de uma emulsão, através de um bocal para uma câmara aquecida formando imediatamente um pó. A rapidez do método e a sua eficácia asseguram a produção de produtos microbiologicamente estáveis, com baixos custos e propriedades específicas (Gharsallaoui et al., 2007; Nedovic et al., 2011). Existem vários exemplos na literatura descrevendo a encapsulação de compostos e extratos bioativos por secagem por spray. Estes incluem extratos brutos (Chiou et al., 2007; Ahmed et al., 2010; Rocha-Gúzman et al., 2010; Sansone et al., 2011; Fernandes et al., 2012b; Krishnaiah et al., 2012; Langrish & Premarajah, 2013; Parthasarathi et al., 2013; Igual et al., 2014; Sansone et al., 2014; Cortés-Rojas et al., 2015), carotenóides (Aissa et al., 2012; Guadarrama-Lezama et al., 2012), enzimas (Bule et al., 2010; Santa-Maria et al., 2012), óleos essenciais (Baranauskienė et al., 2006; Adamiec et al., 2012; Garcia et al., 2012; Almeida et al., 2013; Costa et al., 2013; Cortés-Rojas et al., 2014), ácidos gordos (Shaw et al., 2007; Rubilar et al., 2012; Gallardo et al., 2013; Ng et al., 2013), compostos fenólicos (incluindo antocianinas) (Ersus & Yurdagel, 2007; Saénz et al., 2009; Nayak, Rastogi, 2010; Robert et al., 2010; Tonon et al., 2010; Bakowska-Barczak & Kolodziejczyk, 2011; Sansone et al., 2011; Berg et al., 2012; Osorio et al., 2012; Pillai et al., 2012; Robert et al., 2012; Visentin et al., 2012; Ezhilarasi et al., 2013; Gallegos-Infante et al., 2013; Medina-Torres et al., 2013; Silva et al., 2013; Souza et al., 2013; Sun-Waterhouse et al., 2013; Çam et al., 2014; Pang et al., 2014; Souza et al., 2014) e vitaminas (Romo-Hualde et al., 2012). É também verificado que a grande parte dos materiais de parede usados, tal como previamente referido, são hidratos de carbono e seus derivados. Contudo, Medina-Torres et al. (2013) encapsularam ácido gálico em mucilagem obtida diretamente de *Opuntia ficus* Indica, enquanto Cortés-Rojas et al. (2014) encapsularam eugenol com formulações lipídicas, ambos os estudos com bons resultados e rendimentos de encapsulação elevados. Estes resultados mostram a constante evolução do

presente método e várias possibilidades de ultrapassar as restrições relacionados com o número limitado de materiais de revestimento, tal como foi referido por Gouin et al. (2004).

Os processos que incluem uma etapa de coagulação são também vulgarmente utilizados para encapsular compostos e extratos bioativos para fins alimentares, sendo os mais comuns, os baseados em alginato (Chandy et al., 1998; Deladino et al., 2008; Betancur-Ancona et al., 2011; Wichchukit et al., 2013; Santos et al., 2013; Martins et al., 2014b). As esferas de alginato são formadas a partir de um copolímero poli-iónico obtido a partir de algas marinhas castanhas, sendo frequentemente usado como estabilizante e espessante em muitos produtos alimentares. A sua coagulação pode ser promovida por gelificação externa (p.ex. usando o cloreto de cálcio (fonte de cálcio, ião bivalente) adicionada à solução de coagulação) ou gelificação interna (p.ex. usando o carbonato de cálcio como fonte cálcio adicionado à solução de alginato). No primeiro caso, a gelificação ocorre sobretudo à superfície da cápsula, e no segundo caso no interior das partículas em formação. As esferas formadas, devido ao seu grau de reticulação iónica e funcionalidade, permitem o controlo de absorção de água e, assim, a libertação do bioativo (Goh et al., 2012). A preparação das esferas de alginato, facilmente implementada a nível laboratorial, é muito usada para encapsular uma grande variedade de compostos (hidrofílicos, lipofílicos, óleos entre outros), sendo a libertação controlada conseguida através da modificação do pH (Gouin, 2004; Goh et al., 2012).

A tecnologia de liofilização, que permite a encapsulação de vários constituintes alimentares, é usada comumente para estabilizar compostos e promover uma libertação controlada (Gouin, 2004). É maioritariamente utilizada para encapsular extratos bioativos (Jung et al., 2013), compostos fenólicos (Laine et al., 2008; Sanchez et al., 2011; Ezhilarasi et al., 2013), vitamina C (Spada et al., 2012a; Spada et al., 2012b) e mesmo óleos essenciais (Naik et al., 2014).

De acordo a revisão da literatura realizada, o uso da tecnologia de electrospray para fins alimentares não é muito comum, tendo sido encontrado apenas um único trabalho sobre o tema (Pérez-Masiá et al., 2015). Este, refere-se à encapsulação de ácido fólico (Vitamina B_9) e, de acordo com a descrição fornecida, é uma tecnologia muito apelativa uma vez que não é requerido o uso de solventes orgânicos nem o uso de temperaturas elevadas.

A coacervação é o segundo método de encapsulação mais usado para fins alimentares, não só porque proporciona a obtenção de eficiências de encapsulação elevadas, mas também porque possibilita uma libertação controlada acionada por mecanismos mecânicos, biológicos ou mesmo alterações de temperatura, proporcionando a versatilidade necessária para o desenvolvimento de uma vasta gama de produtos alimentares (Gouin, 2004). Pode ser dividida em coacervação complexa ou simples; a primeira é baseada na complexação de dois polímeros de cargas opostas que irão formar

uma matriz ou revestimento polimérico forte (Qv et al., 2011). Na coacervação complexa, o quitosano é o material de revestimento preferencial, sendo o alginato o mais comumente usado como o polielectrólito de carga oposta (Chandy et al., 1998; Belščak-Cvitanović et al., 2011; Liang et al., 2011; Hui et al., 2013; Martins et al., 2014b). O quitosano apresenta baixa toxicidade, atividade antimicrobiana, biocompatibilidade, mas é essencialmente a sua muco-adesividade que permite uma absorção transmucosal e uma melhor libertação do bioativo (Liang et al., 2011). Na coacervação simples, o polímero, inicialmente solúvel, é precipitado por mudanças de pH ou temperatura (Nazzaro et al., 2012). As proteínas lácteas (Chen et al., 2013; pan et al., 2014) e as pectinas com PGPR (poliglicerol poliricinoleato) (Frank et al., 2012) são alguns exemplos de materiais de revestimento usados na coacervação simples.

Os processos baseados na preparação de emulsões são também muito comuns na encapsulação para fins alimentares. Permite a encapsulação de ingredientes alimentares solúveis em água ou óleo (Nedovic et al., 2011; Nazzaro et al., 2012). As técnicas de emulsão têm sido utilizadas com sucesso na encapsulação de compostos bioativos, incluindo ácidos gordos (Augustin et al., 2011; Averina & Alléman, 2013), vitaminas (Chen & Subirade, 2006), compostos fenólicos (Seok et al., 2003; Chen & Subirade, 2006; Augustin et al., 2011; Betz & Kulozik, 2011; Betz et al., 2012; Vidal et al., 2012; Malik et al., 2014; Pan et al., 2014), antocianinas (Seok et al., 2003; Betz et al., 2012; Frank et al., 2012; Vidal et al., 2012; Averina & Alléman, 2013; Malik et al., 2014; Pan et al., 2014), óleos (Ostertag et al., 2012; Gupta & Ghosh, 2014) e extratos bioativos (Haidong et al., 2011; Hui et al., 2013). Esta etapa está muitas vezes associada a outro processo, na maioria das vezes a processos baseados em *spray-drying*, dando origem a um pó seco que pode ser imediatamente introduzido numa matriz alimentar (Nedovic et al., 2011). De facto, muitos dos processos de encapsulação têm uma fase inicial que implica a preparação de uma emulsão. É por esta razão que não é fácil fazer uma divisão direta das técnicas de encapsulação; efetivamente, existe muitas vezes sobreposição de métodos. Neste trabalho, e dada a importância dos processos baseados em spray, os casos em que a emulsão está associada a técnicas de spray foram incluídos na categoria dos processos baseados em na tecnologia de spray.

As metodologias baseadas em extrusão, ao contrário dos métodos descritos acima, não são muito usuais. Podem ser divididos em extrusão electrostática e co-extrusão. O método de extrusão compreende a passagem do polímero fundido com o bioativo solubilizado por um bocal, ou o polímero fundido e o bioativo por bocais concêntricos, levando à formação de partículas de elevada densidade e com uma eficiência de encapsulação elevada (Kuang et al., 2010; Nedovic et al., 2011). Esta técnica é primariamente usada para a encapsulação de voláteis e condimentos instáveis (Gouin, 2004). Belščak-Cvitanović et al. (2011) e Barbosa-Pereira et al. (2014) demonstraram a

eficiência deste método na encapsulação de compostos fenólicos. A co-extrusão é usada na preparação de microesferas esféricas com um núcleo hidrofóbico (Nedovic et al., 2011), no entanto pode também ser usado na encapsulação de compostos hidrofílicos com alginato, tal como realizado por Piazza & Roversi (2011).

Os lipossomas são maioritariamente usados na área farmacêutica e cosmética, visando a libertação controlada de agentes terapêuticos e a inclusão de estabilizantes em cremes e loções, respetivamente. Na área alimentar, representam um recurso valioso dado as elevadas eficiências de encapsulação, estabilidade e fácil produção (Gouin, 2004). Os lipossomas têm sido utilizados principalmente para estabilizar e aumentar a biodisponibilidade de moléculas bioativas (Barras et al., 2009; Madrigal-Carballo et al., 2010; Gibis et al., 2014; Hasan et al., 2014). Além disso, são também muito utilizados para encapsular compostos pouco solúveis em certos solventes. Coimbra et al. (2011) demonstraram a eficácia dos lipossomas para a encapsulação do resveratrol, ácido cafeico, carvacrol, entre outros (compostos pouco solúveis em água). Enquanto Rasti et al. (2012) aumentaram a estabilidade oxidativa de ácidos gordos polinsaturados por meio da encapsulação por lipossomas.

Os processos baseados em fluídos supercríticos apresentam grandes vantagens para a encapsulação de substâncias lábeis como óleos essenciais, aparecendo quase sempre associados a outras técnicas de encapsulação. Almeida et al. (2013) aplicaram a impregnação em fluído supercrítico para encapsular óleo essencial de orégãos numa matriz de amido, obtendo um produto homogéneo por um processo rápido dado a baixa viscosidade e a elevada difusividade do CO_2 supercrítico. Por outro lado, Santos et al. (2013), usando a extração supercrítica, e Sosa et al. (2011) e Visentin et al. (2012), usando o processo do anti-solvente, aplicaram estas técnicas para encapsular extratos bioativos com elevada eficiência de encapsulação. As grandes vantagens dos fluídos supercríticos estão relacionadas com as suas propriedades físicas como a viscosidade, densidade, poder de dissolução, difusão e transferência de massa. A solubilização do núcleo e do material de revestimento é, portanto, mais rápida sendo a formação da microcápsula facilitada, isto é, a sua formação ocorre a baixas temperaturas e na ausência de água (Gouin, 2004).

Os processos baseados em ultra-sons, como por exemplo a sonificação, são técnicas fiáveis para aplicação alimentar, sendo usadas habitualmente com a dupla função de extração do bioativo e da formação das microcápsulas (Cilek et al., 2012; Mantegna et al., 2012). Por outro lado, Kalogeropoulos et al. (2009) usaram a sonificação para formar complexos de inclusão do extrato de própolis com β-ciclodextrinas.

Apesar de todos os métodos já descritos anteriormente, existem outros métodos de encapsulação que não são comumente usados para fins alimentares. Um exemplo é a técnica de leito fluidizado, uma técnica de microencapsulação para pós. Necessita da

preparação de uma suspensão com o material de revestimento (p.ex. polissacarídeos, proteínas, emulsionantes e gorduras) e subsequente atomização, oferecendo a possibilidade de alcançar uma libertação controlada do material do núcleo mais efectiva, comparativamente a outras tecnologias existentes (Gouin, 2004; Kuang et al., 2010; Nedovic et al., 2011). Li et al. (2007) aplicaram esta tecnologia obtendo integridade e estabilidade do composto do núcleo após um processo de secagem. A inclusão molecular constitui outro processo pouco utilizado; é geralmente referido como um método supramolecular na medida em que a ligação entre o composto encapsulado e o material de revestimento ocorre por pontes de hidrogénio, forças de Van der Waals ou por efeito de entropia hidrofóbica orientada na cavidade de suporte do substrato. As ciclodextrinas e as vitaminas hidrofóbicas são os materiais de revestimento mais usados nas metodologias de inclusão molecular (Gouin, 2004).

Os processos de separação por suspensão rotacional e co-extrusão centrífuga aparecem como métodos de atomização, possivelmente usados em métodos modificados de encapsulação por spray; a diferença está na formação da cápsula, envolvendo a criação de um filme de menores dimensões do que o obtido em atomizadores comuns (Gouin, 2004). Akhtar et al. (2014), mostraram que reduzindo o tamanho da partícula usando um reator de separação por suspensão rotacional para encapsular flavonoides através da técnica da dupla emulsão, obtinha uma maior estabilização das emulsões preparadas. Outros métodos de microencapsulação também pouco usuais no setor alimentar são a co-cristalização (Sardar et al., 2013; López-Córdoba et al., 2014), impressão núcleo-parede (Blanco-Pascual et al., 2014), nanoprecipitação (Wu et al., 2008; Averina & Allémann, 2013), liofilização (Rosa et al., 2013; Rutz et al., 2013), microondas (Abbasi et al., 2009), método da separação de fases (Zheng et al., 2011), metodologia de superfície de resposta (Lee et al., 2013) e método de evaporação do solvente (Prasertmanakit et al., 2009; Kumari et al., 2010).

2.2.1.3. Materiais de encapsulação

Quando se desenha um protocolo experimental para o desenvolvimento de produtos encapsulados (**Figura 9**), a escolha do material de revestimento é um dos passos mais importantes. Este não pode apresentar toxicidade para o organismo, a sua preparação tem que respeitar o meio ambiente e usar solventes verdes (materiais solúveis em água são, assim, preferenciais) e, finalmente, porque determina o comportamento de libertação controlada do bioativo. Parâmetros como o pH, temperatura, presença de sais e força iónica têm também de ser considerados e definidos de acordo com o objetivo final das microcápsulas a desenvolver. Neste trabalho, os materiais encapsulantes foram divididos em quatro categorias (**Tabela 6**) de acordo com a classificação proposta por Kuang et al.

(2010) que os diferencia em materiais solúveis e insolúveis em água e em polímeros e não polímeros. Dentro de cada categoria é ainda possível subdividi-los em hidratos de carbono e seus derivados, proteínas e seus derivados, polímeros sintéticos e outro tipo de materiais. O material de revestimento e a sua estrutura física influenciam fortemente o desenvolvimento do produto; no entanto, existem restrições que impedem a aplicação de alguns materiais em alimentos. Estes têm que ser considerados como geralmente reconhecidos como seguros (GRAS), biodegradáveis e eficientes como barreira protetora entre o núcleo e o meio envolvente. Tanto a UE, através da EFSA, como os EUA, através da FDA, tem regras muito restritas sobre os materiais que podem ser usados para aplicações alimentares (Vos et al., 2009; Nedovic et al., 2011). De uma forma geral, os materiais mais utilizados são os polissacarídeos de origem vegetal (amido e celulose e seus derivados), exsudados e extratos de plantas (gomas, galactomananas, pectinas e oligassacarídeos de soja), extratos marinhos (carragenina e alginato), polissacarídeos de origem animal e flora microbiana (xantano, gelano, dextrano e quitosano) e também proteínas, lípidos e outros (parafina e alguns materiais inorgânicos) (Zuidam et al., 2010). Estes dados estão de acordo com a revisão efetuada, onde pode ser observado que os materiais solúveis em água, tanto polímeros (p. ex: alginato e quitosano) como não polímeros (p. ex: ciclodextrinas) são os mais usados, precedidos pelos polímeros insolúveis em água (p. ex: amido e caseínas) e, finalmente, não polímeros insolúveis em água (p. ex: leticina).

Relativamente à legislação da UE, não é possível efetuar o acesso a uma lista autorizada de materiais pela EFSA para o desenvolvimento de produtos alimentares. Há lacunas na informação, e a lista existente está em construção. Inclui somente aditivos alimentares e fontes de nutrientes, enumerando somente aqueles que não são considerados aditivos (ex. amido) mas sem qualquer referência ao facto de estarem aprovados ou não (Regulation (EC) No 1333/2008). No que respeita aos EUA, a FDA tem uma lista de ingredientes alimentares aprovados que permite às indústrias e aos investigadores o desenho de protocolos de microencapsulação mais adequados para servir o objetivo da indústria alimentar. Apesar dos compostos acima descritos terem sido identificados como os mais usuais em protocolos de microencapsulação, nem todos estão aprovados pela FDA (ou não foram considerados para revisão ou ainda está pendente a sua avaliação). Na **Tabela 6**, e seguindo as diretrizes da FDA, podemos verificar que os materiais aprovados são os seguintes: ácido esteárico, sacarose, amilopectina, amido de milho, caseinato de cálcio, caseína, FHCO (óleo de canola totalmente hidrogenado), PGPR, β-ciclodextrina, etanol, lactose, PEG (polietileno glicol), alginato, quitosano, proteína de soro de leite, celulose, xantano, acetato de celulose, proteína de soja, inulina, pectina e lisozima. Os materiais com avaliação pendente são: lecitina, cafeína, goma-arábica, proteínas do leite e poloxamero. Não existe nunhuma informação disponível para os restantes materiais. É também

necessário perceber que alguma investigação está direcionada para a descoberta de novos materiais de encapsulação, significando que apesar de não estarem na lista da FDA, podem ser adicionados no futuro. Muitos deles são de origem natural como o amido proveniente de sementes de *Araucaria angustifolia* (Bertol.) Kuntze (Spada et al, 2012a; Spada et al., 2012b), extrato de mucilagem de *Opuntia ficus* Indica (Medina-Torres et al., 2013) e fécula de batata-doce gelificada (Park et al., 2014) e, portanto, são necessários estudos adicionais para garantir a segurança destes materiais.

2.2.2. Incorporação de bioativos microencapsulados em matrizes alimentares

2.2.2.1. Extratos bioativos

A importância de utilizar extratos relaciona-se com os efeitos sinergísticos existentes entre os vários componentes presentes nestes, que resultam muitas vezes numa maior bioatividade. A informação relativa à microencapsulação de extratos bioativos provenientes de diferentes plantas ou outras matrizes naturais, obtidos por extração com vários solventes, está sumariada na **Tabela 7**. Os extratos brutos estão presentes de forma significativa nos estudos de microencapsulação, precedidos pelos compostos fenólicos (e também antocianinas), óleos essenciais, vitaminas, proteínas e extratos de gorduras. A grande maioria dos estudos de microencapsulação visando fins alimentares está focada no desenvolvimento da técnica e, por isso, só inclui a definição do material de revestimento, obtenção de uma morfologia adequada para a microcápsula, eficiência de encapsulação, estabilidade e comportamento de libertação. Os estudos relativos ao desenvolvimento de aplicações finais, como por exemplo, testar os compostos microencapsulados em matrizes alimentares reais são pouco representativos. Chiou & Langrish (2007) encapsularam o extracto aquoso bruto de *Hibiscus sabdariffa* L. utilizando fibras extraídas do mesmo fruto como material de revestimento, visando o desenvolvimento de um novo produto nutracêutico valorizando um subproduto normalmente não consumido. Um estudo semelhante foi conduzido por Berg et al. (2012) no qual a pectina (polissacarídeo natural) foi usada como material de encapsulação para proteger as antocianinas extraídas de frutos do género *Vaccinium*, mostrando que a adição de substâncias gelificantes resulta em maiores eficiências de encapsulação. A otimização das metodologias de encapsulação está em constante desenvolvimento, como é o caso dos processos baseados em fluídos supercríticos, que foram utilizados para encapsular extrato de chá verde de folhas de *Camellia sinensis* L. com policaprolactona (PCL), através da coprecipitação pelo uso de um anti-solvente a alta pressão, demonstrando haver uma maior retenção de catequinas nos coprecipitados, e também para encapsular extratos etanólicos de folhas de *Rosmarinus officinalis* L. com proloxamero, com resultados semelhantes (Sosa et al., 2011; Visentin et

al., 2012). Com um objetivo diferente, mas com a intenção de melhorar a encapsulação e libertação de extratos bioativos, Averina & Allémann (2013) desenvolveram micro- e nano-partículas sensíveis ao pH contendo uma fonte de ácidos gordos polinsaturados, nomeadamente óleos extraídos do músculo de *Thymallus baikalensis* Dybowski, sementes de *Pinus sibrica* Du Tour e óleo de peixe comercial, usando as técnicas de difusão-emulsão e nanoprecipitação com resultados promissores. Barras et al. (2009) desenvolveram nanopartículas lipídicas contendo extratos de polifenóis para aumentar a sua solubilidade e estabilidade. Muitos dos estudos com compostos fenólicos foram realizados com o objetivo principal de otimizar os processos de encapsulação (Saénz et al., 2009; Betz & Kulozik, 2011; Sosa et al., 2011; Gibis et al., 2014) usando diferentes tipos de extratos (ex. alcoólicos, aquosos, hidro-alcoólicos, etc.). Efectivamente, não há protocolos estandardizados específicos para a extração de cada classe de compostos fenólicos, estando esta dependente da natureza da amostra e do objetivo do trabalho (conhecimento da estrutura e quantificação) (Santos-Buelga, 2012). Em termos de proteínas (Gharsallaoui et al., 2012; Blanco-Pascual, 2014), vitaminas (Romo-Hualde et al., 2012), fitoesteróis (Ma et al., 2011) e óleos essenciais (Baranauskienė et al., 2006; Garcia et al., 2012; Costa et al., 2013), a maioria dos estudos foram também conduzidos com o objetivo de desenvolver novas metodologias de encapsulação e testar novos materiais, ou para optimizar o processo.

Tabela 7. Extratos bioativos microencapsulados (Dias et al., 2015).

Extratos bioativos	Origem	Solvvente/Método extração	Referência
Extratos de antocianinas	*Bactris guineensis* L. (frutos)	Metanol/ácido acético (19:1, *v/v*)	Osorio et al., 2012
	Daucus carota L. (raízes)	Etanol	Ersus & Yurdagel, 2007
	Euterpe oleracea Mart. (polpa de fruta)	Sumo	Tonon et al., 2010
	Garcinia indica Choisy (polpa de fruta)	Água acidificada	Nayak & Rastogi, 2010
	Myrciaria cauliflora (Mart.) (pele de fruta)	Etanol acidificado	Santos et al., 2013; Silva et al.,2013
	Vaccinium (género de fruta)	*	Betz & Kulozik, 2011; Bert & Bretz, et al., 2012; Frank et al., 2012
Extratos brutos	*Bidens pilosa* L. (partes aéreas)	Etanol	Cortés-Rojas et al., 2015
	Camellia sinensis L. (folhas)	Acetona; etanol	Haidong et al.,2011; Sosa et al., 2011
	Eugenia uniflora L. (frutos)	Sumo	Rutz et al., 2013
	Fadogia ancylantha Schweinf. (partes aéreas)	Etanol/água (70:30, *v/v*)	Sansone et al., 2011
	Garcinia cowa Roxb (frutos)	Água	Parthasarathi et al., 2013
	Hibiscus sabdariffa L. (frutos)	Água	Chiou & Langrish, 2007; Langrish & Premarajah, 2013
	Ilex paraguariensis A. St. Hil. (partes aéreas)	Água	López-Córdoba et al., 2014
	Ipomoea batatas L. Lam variety, Sinjami (tubérculo)	*	Ahmed et al., 2010
	Lippia sidoides Cham. (folhas)	Etanol/água (50:50, *v/v*)	Fernandes et al., 2012b
	Melissa officinalis L. (partes aéreas)	Etanol/água (70:30, *v/v*)	Sansone et al., 2011
	Morinda citrifolia L. (frutos)	Acetato de etilo	Krishnaiah et al., 2012
	Paeonia rockii (S.G.Haw & Lauener) (raízes)	Polar	Sansone et al., 2014
	Cinco ervas: *Paeonia suffruticosa* Andrews, *Phellodendron chinense* Schneid*, Lonicera japónica* Thunb, *Mentha Spicata* L. e *Atractylodes lancea* Thunb.	Água	Hui et al., 2013
	Piper sarmentosum Roxb.	Água	Chan et al., 2010
	Própolis	Etanol	Kalogeropoulos et al., 2009
	Quercus resinosa Liebm. (folhas)	Água	Rocha-guzmán et al., 2010
	Solanum quitoense L. (polpa)	*	Igual et al., 2014
	Tussilago farfara L.	*	Sansone et al., 2011

Extratos brutos de ácidos gordos	Gordura de peixe	Hídrolise	Averina & Alléman, 2013
	Pinus sibirica Du Tour (sementes)	*	Averina & Alléman, 2013
	Thymallus baikalensis Dybowski (músculo)	Etanol	Averina & Alléman, 2013
Óleos essenciais	*Citrus hydrix* D.C. (pele dos frutos)	Água	Adamiec et al., 2012
	Cymbopogon nardus G. (partes aéreas)	*	Baranauskienė et al., 2006
	Majorana hortensis L. (partes aéreas)	*	Baranauskienė et al., 2006
	Origanum vulgare L. (partes aéreas)	*	Baranauskienė et al., 2006
	Origanum vulgare L. (flores e folhas)	Água	Almeida et al., 2013; Costa et al., 2013; Garcia et al., 2014
Ácidos gordos	Comercial	*	Rubilar et al., 2012; Gallardo et al., 2013; Gupta & Ghosh, 2014
	Hibiscus cannabinus L. (sementes)	Hexano	Ng et al., 2013
Extractos de ésteres de fitoesterois	Comercial	*	Ma et al., 2011
Extratos polifenólicos	*Achillea millefolium* L. (partes aéreas)	Água	Belščak-Cvitanović et al., 2011
	Cabernet Sauvignon (frutos)	Sumo (vinho)	Sanchez et al., 2011
	Camellia sinensis L. (folhas)	Etanol	Liang et al., 2011
	Comercial	*	Barras et al., 2009; Barbosa-Pereira et al., 2014; Tavano et al., 2014
	Crategus laevigata (Poir.) Dc. (partes aéreas)	Água	Belščak-Cvitanović et al., 2011
	Glechoma hederacea L. (partes aéreas)	Água	Belščak-Cvitanović et al., 2011
	Hypericum perforatum L. (folhas e flores)	Metanol	Kalogeropoulos et al., 2010
	Ilex paraguariensis A. St. Hil. (partes aéreas)	Água	Deladino et al., 2008
	Myrica, género (frutos)	Etanol	Zheng et al., 2011
	Olea europea L. (folhas)	Água	Belščak-Cvitanović et al., 2011
	Orthosiphon stamineus Benth (folhas)	Metanol/água (50:50, *v/v*)	Pang et al., 2014
	Prunus cerasus L. (bagaço)	Etanol/água (50:50, *v/v*)	Cilek et al., 2012
	Punica granatum L. (frutos)	Etanol e sumo	Robert et al., 2010
	Punica granatum L. (peles)	Água	Çam et al., 2014
	Quercus resinosa Liebm. (folhas)	Água	Gallegos-Infante et al., 2013
	Ribes nigrum L. (bagaço)	Etanol/água/ ácido cítrico (80:20 *v/v*; 5%)	Bakowska-Barczak & Kolodziejczyk, 2011
	Rosmarinus officinalis L.	Etanol	Visentin et al., 2012

	(folhas)		
	Rubus chamaemorus L. (frutos)	Água/acetona (70:30, *v/v*)	Laine te al., 2008
	Rubus idaeus L. (folhas)	Água	Belščak-Cvitanović et al., 2011
	Rubus ulmifolius Schott (flores)	Metanol/água (80:20, *v/v*)	Martins et al., 2014b
	Urtica dioica L. (folhas)	Água	Belščak-Cvitanović et al., 2011
	Vaccinium myrtillus L. (frutos)	*	Betz et al., 2012
	Vitis labrusca L. (sementes e frutos)	Água/etanol (67.6:32.4, *v/v*)	Souza et al., 2014
	Vitis vinifera L. (sementes)	Tampão acetato	Gibis et al., 2014
	Aristotelia chilensis [Molina] Stuntz (folhas)	Etanol/água (40:60, *v/v*)	Vidal et al., 2012
Extratos de polifenóis e betalaínas	*Opuntia ficus* Indica (frutos)	Sumo e etanol	Saénz et al., 2008
Extratos de polifenóis e de gordura	Comercial	*	Coimbra et al., 2012
Extratos de proteínas	Comercial	*	Blanco-Pascual et al., 2014
	Pisum sativum L. (grão)	*	Gharsallaoui et al., 2012
Extratos de vitaminas	*Capsicum annum* L. variedade Piquillo (sementes, peles e caules)	CO_2	Romo-Hualde et al., 2012
Extratos de vitaminas e enzimas	Comercial	*	Stratulat et al., 2014
Extratos d óleos	Comercial	*	Ostertag et al., 2012; Park et al., 2014

*-informação não disponível.

Após otimização do processo de encapsulação, é necessário verificar se o extrato manteve, reduziu ou se aumentou as suas características bioativas. Para o efeito devem ser realizados ensaios de bioatividade para avaliação da atividade antioxidante e antimicrobiana, e quantificar os compostos fenólicos totais. Para avaliar a atividade antioxidante, a atividade captadora de radicais DPPH (2,2-difenil-1-picril-hidrazilo) é o método mais comum, não só para a caracterização da amostra, mas também para avaliar a manutenção da bioatividade. Os estudos realizados por López- Córdoba et al. (2014) e Chan et al. (2010) com extratos brutos de partes aéreas de Ilex paraguarensis A. St. Hil. E Piper sarmentosum Roxb., respetivamente, mostraram que a encapsulação não afetou, positiva ou negativamente, a atividade antioxidante dos extratos. Por outro lado, nos estudos feitos por Igual et al. (2014) e Parthasarathi et al. (2013) com polpa de Solanunm quitoense L. e frutos de Garcinia cowa Roxb., respetivamente, a encapsulação mostrou ser efetiva, uma vez que se observou um aumento na atividade em resultado da proteção contra a degradação. Os extratos de antocianinas obtidos de polpa de frutos de Garcinia indica Choisy (Nayak & Rastogi, 2010), Euterpe oleracea Mart. (Tonon et al., 2010) e raízes de Daucus carota L. (Ersus & Yurdagel, 2007) foram encapsulados com maltodextrinas, que provaram ser eficientes na proteção destes extratos, cuja estabilidade e atividade antioxidante aumentaram após microencapsulação. Com outro objetivo, Deladino et al. (2008) usaram o método do DPPH para avaliar a difusão e a cinética do sistema microencapsulado produzido. A capacidade de absorção dos radicais de oxigénio ("Oxygen radical absorbance capacity"- ORAC) e os ensaios do ácido 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfónico (ABTS) e a capacidade antioxidante em equivalentes de Trolox ("trolox equivalent antioxidant capacity" - TEAC) são também técnicas usados na avaliação da atividade antioxidante de extratos microencapsulados (Bakowska-Barczak & Kolodziejczyk, 2011; Belščak-Cvitanović et al., 2011; Betz et al., 2012; Vidal et al., 2012; Almeida et al., 2013; Langrish & Premarajah, 2013; Silva et al., 2013). Como foi mencionado anteriormente, a quantificação de fenóis totais é também uma metodologia muito comum para avaliar a eficácia do processo de encapsulação (Ahmed et al., 2010; Kalogeropoulos et al., 2010; Robert et al., 2010; Sanchez et al., 2011; Sansone et al., 2011; Krishnaiah et al., 2012; Gallegos-Infante et al., 2013; Ng et al., 2013; Rutz et al., 2013; Martins et al., 2014b; Pang et al., 2014; Cortés-Rojas et al., 2015). Adicionalmente, alguns estudos descrevem o uso de carotenóides para inferir a eficácia do processo de microencapsulação (Rutz et al., 2013; Santos et al., 2013).

As propriedades antibacterianas e antifúngicas estão entre as bioatividades mais estudadas. Tal justifica-se, quer pelo aumento da resistência dos microrganismos aos antibióticos sintéticos comercialmente disponíveis, quer pelo facto de as matrizes naturais apresentarem um elevado potencial para atuar como novos medicamentos. Existem alguns

estudos que focam a microencapsulação de extratos naturais apresentando atividade antibacteriana e antifúngica. Sansone et al. (2014) e Fernandes et al. (2012b) reportaram a atividade antifúngica de raízes de Paeonia rockii (S.G.Haw & Lauener) e de folhas de Lippia sidoides Cham., respetivamente, mostrando a vantagem da sua microencapsulação uma vez que observaram uma melhoria na atividade antifúngica comparativamente ao uso dos extratos na forma livre. A atividade antibacteriana do óleo essencial extraído da pele de frutos de Citrus hydrix D.C. foi avaliada por Adamiec et al. (2012), que também descreveram o incremento da atividade dos extratos microencapsulados. Souza et al. (2014) estudaram o efeito antimicrobiano de extratos etanol/água (67.6% v/v) de Vitis labrusca L. microencapsulados, que demonstraram uma boa atividade inibitória do crescimento de Staphylococcus aureus e Listeria monocytogenes.

Outros resultados apontam melhorias na função óssea em ratos (Haidong et al., 2011) e da citotoxicidade in vitro (Liang et al., 2011) decorrentes do uso de chá de C. sinensis microencapsulado. A atividade antioxidante de extratos aquosos microencapsulados de pele de Punica granatum L., inibidora da α-glucosidase, e o efeito anti-inflamatório de polifenóis comerciais e extratos de óleo foram também descritos (Coimbra et al., 2011; Çam et al., 2014).

Como pode ser observado na Figura 9, os estudos de libertação in vitro constituem uma das etapas mais relevantes aquando do desenvolvimento e validação de um produto microencapsulado. Um sistema de microencapsulação bem-sucedido tem de proteger os compostos bioativos assegurando a manutenção da sua biodisponibilidade, mas também garantir o comportamento de libertação pretendido (temporalmente e orientado para um alvo). Os estudos de libertação in vitro podem ser realizados simulando o ambiente gastrointestinal usando tampões de pH que mimetizam as condições da digestão (Hui et al., 2013; Tavano et al., 2014), ou usando modelos in vitro gastrointestinais contendo enzimas e tampões de pH (Kalegeropoulos et al., 2009; Zheng et al., 2011; Frank et al., 2012; Park et al., 2014). Tavano et al. (2014) mostraram, mediante estudos de libertação in vitro, que a curcumina e a quercetina microencapsuladas em niossomas apresentavam uma melhor solubilidade após digestão gastrointestinal. Frank et al. (2012) e Park et al. (2014) reportaram que após digestão gastrointestinal in vitro, os extratos de antocianinas extraídas de V. myrtillus L. e óleo comercial microencapsulados, respetivamente, apresentavam alta resistência a mudanças no pH durante a digestão, sendo somente libertados nas condições intestinais. Isto corrobora o interesse e a eficácia da microencapsulação no desenho adequado de sistemas de libertação para compostos, solúveis ou insolúveis em água, para serem incorporados em produtos alimentares inovadores.

2.2.2.2. Compostos bioativos

A importância do estudo de compostos bioativos puros assenta no facto de estes terem uma bioatividade elevada, tendo também várias aplicações, incluindo no setor da indústria alimentar e farmacêutica. Neste contexto, o seu isolamento da matriz original é um tema de estudo interessante e que confere valor acrescentado aos produtos desenvolvidos. Na **Tabela 8**, descreve-se um conjunto de compostos bioativos microencapsulados para aplicações alimentares. O número de artigos referentes à encapsulação de compostos puros é marcadamente inferior à dos extratos bioativos. No entanto, os compostos fenólicos são uma vez mais, as moléculas individuas mais usadas nos estudos de microencapsulação. A grande maioria dos estudos está focada no desenvolvimento e optimização da técnica de microencapsulação (Kumari et al., 2010; Mantegna et al. 2012; Lee et al., 2013; Rosa et al., 2013; Silva et al., 2013; Souza et al., 2013; Bagheri et al., 2014), incluindo o teste de novos materiais de encapsulação. Um exemplo é o trabalho realizado por Medina-Torres et al. (2013) no qual o ácido gálico comercial foi encapsulado usando mucilagem extraída de *O. ficus* Indica. Robert et al. (2012) encapsularam também ácido gálico usando amido acetilado e inulina, obtendo uma eficiência de encapsulação superior com o primeiro material. Por outro lado, para os compostos fenólicos quercetina e vanilina, o uso da inulina resultou melhores resultados (Sun-Waterhouse et al., 2013). Apesar dos efeitos benéficos dos compostos fenólicos, a sua estabilidade e biodisponibilidade ficam altamente comprometidas durante o processamento alimentar, armazenamento e digestão, como foi previamente mencionado. Por isso, a microencapsulação dos compostos fenólicos puros pode providenciar uma via para manter ou aumentar a sua atividade antioxidante (Wu et al., 2008; Malik et al., 2014), estabilidade (Laine et al., 2008; Sansone et al., 2011) e biodisponibilidade (Jung et al., 2013; Hasan et al., 2014). A atividade antimicrobiana foi também testada em microcápsulas contendo ácido clorogénico isolado de folhas de *Nicotiana tabacum* L., indicando que a atividade não foi afetada pela microencapsulação, constituindo uma alternativa no desenvolvimento de produtos com propriedades antimicrobianas (Zhao et al., 2010).

Os ácidos gordos polinsaturados foram também alvo de estudos de microencapsulação. Os seus efeitos benéficos reconhecidos para a saúde tornam estes compostos muito apelativos para o enriquecimento de matrizes alimentares. Contudo, a sua natureza lipofílica e a tendência para a rancificação constituem obstáculos ao desenvolvimento de sistemas de libertação eficientes. Naik et al. (2014) desenvolveram uma técnica de encapsulação para a encapsulação de ácido α-linoleico isolado de sementes de *Lepidium sativum* Linn. usando a liofilização para conseguir um composto estável e biodisponível. Por outro lado, Shaw et al. (2007) e Rasti et al. (2012) desenvolveram

sistemas lipofílicos diferentes para encapsular ácidos gordos ω-3 comerciais. Shaw et al. (2007) aplicaram a técnica de *spray-drying* com lecitina e quitosano como material de revestimento, para prevenir a oxidação lipídica, demostrando a grande potencialidade deste sistema multicamada. Rasti et al. (2012) usaram sistemas baseados em lipossomas para encapsular ácidos gordos ω-3, usando fosfolípidos de soja como material de revestimento. Estes autores demonstraram que a formação dos lipossomas em meio aquoso, combinado com a proteção antioxidante dos fosfolípidos, aumentava a estabilidade e prevenia a peroxidação dos ácidos gordos. Outros compostos, também muito instáveis e, que por isso, beneficiam com a aplicação de técnicas de microencapsulação são os óleos essenciais e seus constituintes.

Tabela 8. Compostos bioativos individuais microencapsulados (Dias et al., 2015).

Classe	Compostos bioativos individuais	Origem	Referência
Carotenóides	Curcumina	Comercial	Hasan et al., 2014; Malik et al., 2014; Xu et al., 2014
	Luteína	Comercial	Qv et al., 2011
	β-caroteno	Comercial	Spada et al., 2012a; Spada et al., 2012b; Cortés-Rojas et al., 2015
	β-caroteno	*Capsicum annuum* L. (frutos)	Guadarrama-Lezama et al., 2012
Carotenóides e vitaminas	Curcumina e retinol	Comercial	Pan et al., 2014
Enzimas	Celulases e xilanases	Comercial	Santa-Maria et al., 2012
	Coenzima Q10	Comercial	Bule et al., 2010
Óleos essencias	Oleoresina de cardamomo	Comercial	Sardar et al, 2013
	Eugenol e acetato de eugenilo	*Syzygium aromaticum* L. (rebentos)	Cortés-Rojas et al., 2014
Ácidos gordos	Ácido α-linolênico	*Lepidium sativum* Linn. (sementes)	Naik et al., 2014
	Ácidos gordos ω-3	Comercial	Rasti et al., 2012; Rubilar et al., 2012
Compostos fenólicos	Cafeína	Comercial	Bagheri et al., 2014
	Catequinas	*Camellia sinensis* L. (folhas)	Jung et al., 2013
	Ácido clorogénico	*Nicotiana tabacum* L. (folhas)	Zhao et al., 2010
	Ácido elágico	Comercial	Madrigal-Carballo et al., 2010
	Ácido gálico	Comercial	Robert et al., 2012; Medina-Torres et al., 2013; Rosa et al., 2013
	Isoflavona	Comercial	Seok et al., 2003
	Mangiferina	*Mangifera indica* L. (casca)	Souza et al., 2013
	Naringenina e quercetina	Comercial	Sansone et al., 2011
	Quercetina	Comercial	Wu et al., 2008
	Quercetina e vanilina	Comercial	Sun-Waterhouse et al., 2013
	Quercitrina	*Albizia chinensis* L. flores (90:10, *v/v*)	Kumari et al., 2010
	Resveratrol	*Arachis hypogaea* L. broto	Lee et al., 2013
	Resveratrol	*Polygonum cuspidatum* Siebold & Zucc roizes	Mantegna et al., 2012
	Rutina e antocaaninas	*Hibiscus sabdariffa* L. calli seco	Akhtar et al., 2014
Proteínas	Albumina e hirudina	Comercial	Chandy et al., 1998
	Papaina	Comercial	Betancur-Ancona et al., 2011
Ácidos orgânicos	Ácido cítrico	Comercial	Piazza & Roversi, 2011
	Ácido (−)-hidroxicítrico	*Garcinia cowa* Roxb frutos	Abbasi et al., 2009
Compostos organosulfurados	Alicina	*Allium sativum* L. dentes sem casca	Pillai et al., 2012; Ezhilarasi et al., 2013a; Ezhilarasi et al., 2013b
Vitaminas	Ácido fólico (Vitamina B_9)	Comercial	Li et al., 2007
	Riboflavina (Vitamina B_2)	Comercial	Piazza & Roversi, 2011
Misturas de bioativos	Gordura de peixe, resveratrol, tributirina	Comercial	Piazza & Roversi, 2011
	Glucose, vitamina B_{12}, azeite de oliva	Comercial	Prasertmanakit et al., 2009; Pérez-Masiá et al., 2015
	Gordura de peixe, fitoesterós (5α-colestano, β-	Comercial	Chen & Suribade, 2006; Wichchukit et al., 2013

	sitosterol, campesterol e estigmasterol) e limoneno

Para além da sua natureza lipofílica, os óleos essenciais são também compostos voláteis que necessitam da proteção oferecida pela microencapsulação. Neste contexto, os transportadores lipídicos que envolvem formulação de soluções contendo lípidos sólidos, surfactantes e compostos para secagem (p. ex: polissacarídeos) têm conduzido a altas eficiências de encapsulação para o eugenol e acetato de eugenilo isolados de gomos de *Syzygium aromaticum* L. (Cortés-Rojas et al., 2014). A microencapsulação por co-cristalização de oleorresina de cardamomo proporcionou proteção aos seus componentes maioritários, 1,8-cineol e acetato de α-terpinilo; tendo ocorrido, no entanto, alguma degradação durante o processo de embalamento e armazenamento (Sardar et al., 2013).

Os carotenóides são uma família de compostos muito utilizados como corantes alimentares em substituição de corantes sintéticos, apresentando adicionalmente efeitos antioxidantes e antiangiogénicos. No entanto, têm uma grande tendência para a oxidação e isomerização. Qv et al. (2011) e Xu et al. (2014) estudaram a estabilidade da luteína e curcumina, após microencapsulação por coacervação complexa com Ca-alginato/k-carragenina e Ca-alginato/lisozima, respetivamente. Ambos os processos originaram eficiências de encapsulação elevadas e demonstraram a eficácia do método usado. Spada et al. (2012a; 2012b) microencapsularam β-caroteno comercial em amido modificado obtido de sementes de *Araucaria angustifolia* (Bertol.) Kuntze concluíram que a gelificação do amido conduziu uma eficiência de encapsulação superior para o carotenoide, assegurando a sua proteção em condições adversas. Aissa et al. (2012) testaram microcápsulas enriquecidas com β-caroteno quanto aos seus efeitos genotóxicos e antiangiogénicos, usando goma-arábica como material de revestimento. Os autores verificaram a preservação dos efeitos genotóxicos, contudo um decréscimo na atividade antiangiogénica, provavelmente devida à perda de biodisponibilidade.

Outros exemplos de compostos individuais que têm sido alvo de estudos de microencapsulação incluem ácidos orgânicos (Abbasi et al., 2009; Pillai et al., 2012; Ezhilarasi et al., 2013a; Ezhilarasi et al., 2013b), enzimas (Bule et al., 2010; Santa-Maria et al., 2012) e proteínas (Chandy et al., 1998; Betancur-Ancona et al., 2011).

A vitamina B_2 (riboflavina) e a vitamina B_9 (ácido fólico) foram também encapsuladas para fins alimentares. Devido aos seus efeitos benéficos reconhecidos para a saúde, mas elevada tendência para a degradação e perda de biodisponibilidade, têm sido alvo de estudos de libertação *in vitro* onde são avaliados novos sistemas de libertação. Chen & Subirade (2006) testaram a libertação de riboflavina simulando fluídos gástricos, intestinais e pancreáticos, concluindo que as microcápsulas de riboflavina compostas por alginato/proteína de soro de leite são semidestruídas pelos fluídos intestinais, ocorrendo a libertação completa no fluido pancreático. Para estimar o tempo de prateleira de um produto, Wichchukit et al. (2013) estudaram a libertação da riboflavina incorporada num produto

alimentar, uma bebida modelo. Prasertmanakit et al. (2009) estudaram a libertação *in vitro* de ácido fólico em microcápsulas de acetato de celulose, material que origina uma boa eficiência de encapsulação. A adição de um glúcido solúvel em água, a sacarose, originou o inchamento da matriz polimérica, permitindo um melhor controlo na libertação do ácido fólico.

Uma progresso no desenvolvimento de sistemas de libertação controlada consiste no encapsulamento de misturas de compostos bioativos dentro de uma mesma microcápsula, obtendo-se assim vários efeitos benéficos. Augustin et al. (2011) desenvolveram uma emulsão óleo-em-água para estabilizar gordura de peixe comercial, resveratrol e tributirina, usando caseinato, glucose e amido. Estudaram o seu comportamento no trato gastrointestinal, tendo obtido uma maior biodisponibilidade para todos os compostos. Pan et al. (2014) estudaram a estabilidade oxidativa da curcumina (carotenóide) e retinol (óleo essencial) em emulsões óleo-em-água, com resultados muito satisfatórios.

2.2.2.3. Incorporação em matrizes alimentares

Alguns exemplos de estudos de desenvonvimento de aplicações finais envolvendo extratos bioativos ou compostos puros isolados estão descritos na **Tabela 9**. Após uma ampla revisão da literatura, confirmou-se que a vasta maioria dos estudos não incluem a validação dos bioativos microencapsulados por incorporação em matrizes alimentares. Apenas doze estudos incluíram este passo final, crucial para a indústria alimentar. No geral, o leite e os derivados lácteos como queijo, iogurtes e gelados são as matrizes preferenciais focadas nestes estudos. O setor dos cereais, pão e massas têm também um peso significativo nos estudos de desenvolvimento de aplicações finais. O chá, sopa e carne foram também matrizes testadas para a incorporação de microcápsulas contendo bioativos. Os extratos fenólicos da pele de *Punica granatum* L. foram estudados por Çam et al. (2014) e incorporados em gelado para aumentar a sua atividade antioxidante inibidora da α-glucosidade. Martins et al. (2014b) e Robert et al. (2010) incorporaram extratos fenólicos de flores de *Rubus ulmifolius* Schott. e frutos de *Punica granatum* L., respetivamente. Martins et al. (2014b) obtiveram maior atividade antioxidante nos iogurtes incorporados com extratos microencapsulados, comparativamente à utilização dos extratos na forma livre e controlo (iogurte sem extrato); por outro lado Robert et al. (2010) também observaram um maior conteúdo de compostos fenólicos e antocianinas no iogurte enriquecido com extratos microencapsulados. A técnica de incorporação desenvolvida por Barbosa-Pereira et al. (2012) na qual extratos fenólicos foram adicionados a embalagens ativas visando o aumento do tempo de prateleira de produtos à base de carne, apontou para resultados promissores na retardação da oxidação lipídica e crescimento microbiano. Em termos de compostos fenólicos puros, uma isoflavona solúvel em água foi emulsionada com poliglicerólico

monoestearato e, posteriormente, incorporada em leite para estudar a sua estabilidade durante o armazenamento e após digestão *in vitro*. Foi demonstrado que a isoflavona microencapsulada não afetou o sabor do leite e que a sua absorção no intestino aumentou (Seok et al., 2003). O ácido cítrico e seu derivado, ácido (-)-hidroxicítrico, foram também incorporados; em particular, o composto derivado extraído dos frutos de *Garcinia cowa* Roxb. foi incorporado em pão (Ezhilarasi et al., 2013a, Ezhilarasi et al., 2013b) e em massa (Pillai et al., 2012); em ambos os casos o pão e massa enriquecidos com os bioativos microencapsulados mostraram bons atributos sensoriais, o que prova a viabilidade de usar este tipo de estratégia no desenvolvimento de produtos alimentares. O ácido cítrico, numa escala micronizada, foi também incorporado em pastilha elástica, usando uma técnica baseada em caseína e inulina para formar as microcápsulas bioativas, para obter pastilhas com propriedades promotoras de saúde (Abbasi et al., 2009). A sopa, um dos produtos mais consumidos mundialmente, serviu também como matriz para estudos de incorporação realizados por Rubilar et al. (2012). Foram adicionadas microcápsulas contendo ácidos gordos (óleo de linhaça) a sopa instantânea para desenvolver um novo produto funcional; adicionalmente, e uma vez que o óleo de linhaça foi incorporado numa matriz polimérica contendo goma-arábica e maltodextrina, conseguiu-se um maior controlo da libertação do núcleo lipofílico. Sardar et al. (2013) encapsularam um composto lipofílico, oleorresina de cardamomo, usando sacarose como material de revestimento e o método de co-cristalização, dando origem a cubos de açúcar condimentados chás. Os cubos produzidos mantiveram-se estáveis durante o armazenamento quando embalados num laminado metalizado de três camadas.

O queijo, apesar de muito apreciado pelos consumidores, é rico em gordura tendo vindo a ser feitos esforços visando a adição de gorduras de origem vegetal a esta matriz. No entanto, os óleos degradam-se muito rapidamente, beneficiando assim da adição de antioxidantes como as vitaminas A e E, e coenzimas. Neste contexto, o trabalho de Stratulat et al. (2014) teve como intenção inibir a peroxidação lipídica (rancidificação), formulando emulsões, contendo vitaminas A e E, e coenzimas Q_{10}, estabilizadas com caseinato de cálcio. Os resultados mostraram que os óleos vegetais não afetaram a estabilidade do queijo, tendo aumentando assim a presença de antioxidantes.

Tabela 9. Exemplos de estudos com extratos bioativos microencapsulados ou compostos individuais incorporados em matrizes alimentares (Dias et al., 2015).

Matriz alimentar	Bioativo	Origem	Método de encapsulação	Material de encapsulação	Referências
Carne	Extratos fenólicos	Resíduos da indústria cervejeira	Extrusão	Acetato de etileno vinilo e LDPE	Barbosa-Pereira et al., 2014
Chá	Oleoresina de cardamomo	Comercial	Co-cristalização	Sacarose	Sardar et al., 2013
Gelado	Extratos fenólicos	*Punica granatum* L. (peles)	Spray-secagem	Maltodextrina	Çam et al., 2014
Iogurte	Extratos fenólicos	*Rubus ulmifolius* Schott (flores)	Atomização/coagulação	Alginato	Martins et al., 2014
	Extratos fenólicos	*Punica granatum* L. (frutos)	Spray-secagem	Maltodextrina ou proteinas de soja	Robert et al., 2010
Leite	Isoflavona	Comercial	Emulsão	Poliglicerólico monoestearato	Seok et al., 2003
Massa	Ácido (−)-hidroxicítrico	*Garcinia cowa* Roxb. (frutos)	Spray-secagem	Proteinas do soro de leite	Pillai et al., 2012
Pão	Ácido (−)-hidroxicítrico	*Garcinia cowa* Roxb (pele frutos)	Spray-secagem	Proteinas do soro de leite e maltodextrina	Ezhilarasi et al., 2013a
	Ácido (−)-hidroxicítrico	*Garcinia cowa* Roxb (pele frutos)	Liofilização	Proteinas do soro de leite e maltodextrina	Ezhilarasi et al., 2013b
Pastilha elástica	Ácido cítrico	Comercial	Microondas	Caseína e inulina	Abbasi et al., 2009
Queijo	Vitaminas E e A; Coenzima10	Comercial	Emulsão	Caseinato de cálcio	Stratulat et al., 2014
Sopa	Ácidos gordos (óleo de linhaça)	Comercial	Spray-secagem	Goma-arábica e maltodextrina	Rubilar et al., 2012

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3.

Composição química e propriedades bioativas de matrizes vegetais provenientes do Nordeste de Portugal: *Achillea millefolium* L., *Fragaria vesca* L., *Laurus nobilis* L. e *Taraxacum* set. Ruderalia

O capítulo 3 compreende 8 artigos resultantes da atividade experimental associada à caraterização nutricional e química das plantas bem como à avaliação das propriedades bioativas dos seus extratos aquosos e metanol: água (80:20, *v/v*) e ainda a estudos de bioacessibilidade de minerais.

3.1. Achillea millefolium L.

Neste sub-capítulo apresenta-se a caraterização nutricional e química, e as propriedades antioxidantes e citotóxicas de *Achillea millefolium* L. silvestre e comercial e das respetivas infusões, decocções e extratos metanol: água.

3.1.1. Composição química de *Achillea millefolium* L. silvestre e comercial e bioatividade dos extratos metnólicos, infusões e decocções

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Chemical composition of wild and commercial *Achillea millefolium* L. and bioactivity of the methanolic extract, infusion and decoction.

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Abstract

Medicinal plants used in folk medicine are being increasingly studied and used on pharmaceutical, food and nutraceutical fields. Herein, wild and commercial samples of *Achillea millefolium* L. (yarrow) were chemically characterized with respect to their macronutrients, free sugars, organic acids, fatty acids and tocopherols. Furthermore, *in vitro* antioxidant properties (free radicals scavenging activity, reducing power and lipid peroxidation inhibition) and antitumour potential (against breast, lung, cervical and hepatocellular carcinoma cell lines) of their methanolic extract, infusion and decoction (the most consumed forms) was evaluated and compared to the corresponding phenolic profile obtained by high performance liquid chromatography and mass spectrometry. Data obtained showed that the chemical profiles of wild and commercial samples, and also their methanolic extract, infusion and decoction were similar, varying only in the quantities found. Commercial yarrow have higher content of fat and saturated fatty acids, proteins, ash, energy value,

sugars and flavonoids, while the wild sample revealed higher levels of carbohydrates, organic acids, unsaturated fatty acids, tocopherols and phenolic acids. The heterogeneity among the antioxidant and antitumour results of the samples and some low correlations with total phenolic compounds indicates that specific compounds, rather than the totality of them, are involved in the bioactive properties of samples.

Keywords: Achillea millefolium L.; Wild/commercial; Chemical composition; Bioactive properties; Phytochemicals

3.1.1.1. Introduction

In a society increasingly concerned with health and nutrition, medicinal plants emerge as alternative to synthetic products, used not only in traditional medicine but also in a number of food and pharmaceutical products, due to their nutritional properties and bioactivity (Phillipson, 2007). *Achillea millefolium* L., commonly known as yarrow, belongs to Asteraceae family and it is very common in mountain meadows, pathways, crop fields and homegardens. Its infusion or alcohol extract is widely used in Europe as a remedy to treat digestive problems, diabetes, hepato-biliary diseases and amenorrhea, and also consumed for its antitumour, antimicrobial, anti-inflammatory and antioxidant properties, among others (Baretta et al., 2012; Candan et al., 2010; Carvalho, 2010, Cavalcanti et al., 2006; Dall'Acquaa, Bolegob, Cignarellab, Gaionb, & Innocentia, 2011; Jonsdottir, Omarsdottird, Vikingssona, Hardardottirc, & Freysdottir, 2011; Potrich et al., 2010; Trumbeckaite et al., 2011). The decoction is used for digestive and intestinal disorders, but it is also used externally for skin and mucosa inflammations (Rauchensteiner, Nejati & Saukel, 2004). Due to all of these features, yarrow is a good candidate for functional food or nutraceuticals source such as other plants from Asteraceae family: *Chamaemelum nobile* L. (Guimarães et al., 2013a), *Baccharis dracunculifolia* DC. (Guimarães et al., 2013) or *Echinacea angustifolia* DC. (Stefano, Nicola, Fabrizio, Valentina, & Gabbriella, 2010).

Antioxidant properties of *A. millefolium* have previously been reported in hydroalcoholic, methanolic and aqueous extracts, as also in the essential oil (Candan et al., 2010; Kintzios, Papageorgiou, Yiakoumettis, Baričevič, & Kušar, 2010; Trumbeckaite et al., 2011; Vitalini et al., 2011), but not in the infusion or decoction, the most consumed form. Cytotoxicity against human tumour cell lines was also only evaluated with the ethanolic extract (Ghavami, Sardari, & Shokrgozar, 2010) and was related to the presence of sesquiterpene lactones and flavonols (Csupor-Löffler et al., 2009). Antioxidant molecules such as tocopherols and ascorbic acid were quantified in *A. millefolium* and found to be present in considerable amounts (Chanishvili, Badridze, Rapava, & Janukashvili, 2007).

Flavonoids, apigenin and quercetin, and the phenolic acid, caffeoylquinic acid, were reported as the major phenolic compounds present in yarrow plant (Benedek, Gjoncaj, Saukel, & Kopp, 2007; Benetis, Radušienė, & Janulis, 2008; Radušienė, 2011; Vitalini et al., 2011). The above mentioned compounds have the capacity to function as reducing agents, hydrogen donators or singlet oxygen quenchers against reactive species involved in oxidative stress, the main cause for cell death (Carocho & Ferreira, 2013).

The main objective of the present work was to compare chemical composition of wild and commercial *A. millefolium* regarding macronutrients, free sugars, organic acids, fatty acids and tocopherols. Furthermore, *in vitro* antioxidant properties (free radicals scavenging activity, reducing power and lipid peroxidation inhibition) and antitumour potential (against breast, lung, cervical and hepatocellular carcinoma cell lines) of their methanolic extract, infusion and decoction (the most consumed forms) were evaluated and compared to the corresponding phenolic profile.

3.1.1.2. Materials and methods

Samples

The wild yarrow (inflorescences and upper leaves) was collected in Cova de Lua, Bragança, Portugal from 50 plants growing in two different grasslands of about one hectare. The gathered material was mixed, made into a unique sample and further lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA). A voucher specimen was deposited at the Herbarium of the Escola Superior Agrária de Bragança (BRESA). The commercial yarrow was purchased from a local company, Ervital from Castro Daire, Portugal, which produces Mediterranean herbs using organic principles and methods. Each sample was reduced to a fine dried powder (20 mesh) and mixed to obtain homogenate sample.

Standards and Reagents

Acetonitrile (99.9%), n-hexane (95%) and ethyl acetate (99.8%) were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and the fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) were purchased from Sigma (St. Louis, MO, USA), as well as other individual fatty acid isomers, L-ascorbic acid, tocopherol, sugar and organic acid standards. Phenolic standards were from Extrasynthèse (Genay, France). Racemic tocol (50 mg/mL), was purchased from Matreya (Pleasant Gap, PA, USA). 2,2-Diphenyl-1- picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Fetal bovine serum (FBS), L-glutamine, hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic

acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, UT, USA). Acetic acid, ellipticine, sulphorhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (Saint Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Chemical composition of wild and commercial samples

Macronutrients. The samples were analysed for proteins, fat, carbohydrates and ash using the AOAC (1995) procedures. The crude protein content (N×6.25) of the samples was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600±15 ^{o}C. Total carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein) + 3.75 × (g carbohydrate) + 9 × (g fat).

Sugars. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI), after an extraction procedure previously described (Guimarães et al., 2013a) using melezitose as internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD, USA) and an RI detector (Knauer Smartline 2300, Berlin, Germany). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer, Berlin, Germany) operating at 30 ºC (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight.

Organic acids. Organic acids were determined following a procedure previously described (Pereira, Barros, Carvalho, & Ferreira, 2013). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C_{18} column (5 μm, 250 mm × 4.6 mm i.d.) thermostatted at 35 ºC. The elution was performed with sulphuric acid (3.6 mM) using a flow rate of 0.8 mL/min. Detection was carried out in a PDA, using 215 and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm with calibration

curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

Fatty acids. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID)/capillary column as described previously (Guimarães et al., 2013a). The analysis was carried out with a DANI model GC 1000 instrument (Contone, Switzerland), equipped with a split/splitless injector, a flame ionization detector (FID at 260 ºC) and a Macherey–Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 µm d_f).. The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30 ºC/min ramp to 125 ºC, 5 ºC/min ramp to 160 ºC, 20 ºC/ min ramp to 180 ºC, 3 ºC/min ramp to 200 ºC, 20 ºC/min ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the CSW 1.7 Software (DataApex 1.7) and expressed in g/100 g fat.

Tocopherols. Tocopherols were determined following a previously described procedure (Guimarães et al., 2013a). Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco, Easton, MD, USA) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II (250 mm × 4.6 mm i.d.) normal-phase column from YMC Waters (Dinslaken, Germany) operating at 30 ºC. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min, and the injection volume was 20 µL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on calibration curves obtained from commercial standards of each compound using the internal standard (IS) methodology; racemic tocol was used as IS. The results were expressed in mg per 100 g of dry weight.

Bioactivity and phenolic profile of the methanolic extract, infusion and decoction

Samples preparation. The methanolic extract was obtained from the lyophilized wild and commercial plant material. Each sample (1 g) was extracted twice by stirring with 30 mL of methanol (25 ºC at 150 rpm) for 1 h and subsequently filtered through a Whatman No. 4 paper. The combined methanolic extracts were evaporated at 40 ºC (rotary evaporator Büchi R-210, Flawil, Switzerland) to dryness.

For infusion preparation the lyophilized plant material (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under

reduced pressure. For decoction preparation the lyophilized plant material (1 g) was added to 200 mL of distilled water, heated (heating plate, VELP scientific) and boiled for 5 min. The mixture was left to stand for 5 min and then filtered under reduced pressure. The obtained infusions and decoctions were frozen and lyophilized.

Methanolic extracts and lyophilized infusions and decoctions were redissolved in *i)* methanol and water, respectively (final concentration 2.5 mg/mL) for antioxidant activity evaluation, *ii)* water (final concentration 8 mg/mL) for antitumour potential evaluation; and *iii)* water:methanol (80:20, *v/v*) and water, respectively (final concentration 1 mg/mL) for phenolic compounds identification and quantification. The final solutions were further diluted to different concentrations to be submitted to distinct bioactivity evaluation in *in vitro* assays. The results were expressed in *i)* EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) for antioxidant activity, or *ii)* GI_{50} values (sample concentration that inhibited 50% of the net cell growth) for antitumour potential. Trolox and ellipticine were used as positive controls in antioxidant and antitumour activity evaluation assays, respectively.

Antioxidant activity. DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} to Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Guimarães et al., 2013b).

Antitumour potential and cytotoxicity in non-tumour liver primary cells. Five human tumour cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine (MCF-7, NCI-H460 and HCT-15) or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100

mg/mL streptomycin (HeLa and HepG2 cells), at 37 ºC, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT-15 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates. Sulphorhodamine B assay was performed according to a procedure previously described by the authors (Guimarães et al., 2013b).

For hepatotoxicity evaluation, a cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, according to an established procedure (Guimarães et al., 2013b); it was designed as PLP2. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and commercial in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin.

Phenolic profile. Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA) as previously described by the authors (Rodrigues et al., 2012). Double online detection was carried out in the diode array detector (DAD) using 280 and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were characterized according to their UV, mass spectra, retention times, and comparison with authentic standards when available. For quantitative analysis, a 5-level calibration curve was obtained by injection of known concentrations (2.5-100 μg/mL) of different standards compounds: apigenin-6-*C*-glucoside (y=246.05x-309.66; R^2=0.9994); apigenin-7-*O*-glucoside (y=159.62x+70.50; R^2=0.999); caffeic acid (y=611.9x-4.5733; R^2=0.999); 5-*O*-caffeoylquinic acid (y=313.03x-58.20; R^2=0.999); kaempferol-3-*O*-glucoside (y=288.55x-4.05; R^2=1); kaempferol-3-*O*-rutinoside (y=239.16x-10.587; R^2=1); luteolin-6-*C*-glucoside (y=508.54x-152.82; R^2=0.997); luteolin-7-*O*-glucoside (y=80.829x-21.291; R^2=0.999); quercetin-3-*O*-glucoside (y=253.52x-11.615; R^2=0.999) and quercetin-3-*O*-rutinoside (y=281.98x-0.3459; R^2=1). The results were expressed in mg per g of methanolic extract and lyophilized infusion or decoction.

Statistical analysis

For wild and commercial plant material, three samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 18.0 program.

3.1.1.3. Results and Discussion

Chemical composition of wild and commercial samples

The chemical composition of wild and commercial *A. millefolium* in macronutrients, free sugars and organic acids is presented in **Table 10.**

Carbohydrates, followed by proteins, were the major macronutrients in both samples. The commercial sample revealed higher contents of all the macronutrients except in carbohydrates which were higher in the wild yarrow. Fructose, glucose, sucrose and trehalose were found in both samples, while raffinose was only detected in the wild sample. Commercial sample also showed the highest levels of individual and total free sugars. Wild sample presented the highest content in total organic acids, mainly oxalic, quinic, and citric; succinic acid was not detected in the commercial sample and fumaric acid was only found in traces (**Table 10**).

Table 10. Chemical composition of wild and commercial *Achillea millefolium* L. in macronutrients, free sugars and organic acids.

	Wild sample	**Commercial sample**
Fat (g/100 g dw)	5.20 ± 0.13^{b}	8.03 ± 0.00^{a}
Proteins (g/100 g dw)	12.53 ± 0.85^{b}	19.53 ± 0.05^{a}
Ash (g/100 g dw)	6.43 ± 0.11^{b}	8.54 ± 0.88^{a}
Carbohydrates (g/100 g dw)	75.84 ± 0.76^{a}	63.90 ± 0.86^{b}
Energy (kcal/100 g dw)	400.28 ± 0.21^{b}	405.99 ± 3.52^{a}
Fructose	1.11 ± 0.02^{b}	1.31 ± 0.06^{a}
Glucose	0.66 ± 0.04^{b}	1.43 ± 0.08^{a}
Sucrose	0.80 ± 0.03^{a}	0.95 ± 0.11^{a}
Trehalose	0.42 ± 0.04^{b}	1.18 ± 0.17^{a}
Raffinose	0.15 ± 0.00	nd
Total sugars (g/100 g dw)	3.14 ± 0.08^{b}	4.86 ± 0.29^{a}
Oxalic acid	1.08 ± 0.06^{a}	0.92 ± 0.01^{b}
Quinic acid	0.69 ± 0.03^{b}	1.50 ± 0.08^{a}
Malic acid	1.64 ± 0.04^{a}	0.77 ± 0.13^{b}
Shikimic acid	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}
Citric acid	0.83 ± 0.03^{b}	1.25 ± 0.13^{a}
Succinic acid	0.27 ± 0.03	nd
Fumaric acid	0.03 ± 0.00	tr
Total organic acids (g/100g dw)	4.55 ± 0.10^{a}	4.46 ± 0.19^{b}

nd- not detected; dw- dry weight. In each row different letters mean significant differences ($p<0.05$).

Up to twenty-nine fatty acids were identified on wild and commercial *A. millefolium* (**Table 11)**. In both samples linoleic acid (C18:2n-6, PUFA) was the major fatty acid, followed by palmitic acid (C16:0, SFA) in the case of commercial sample, and oleic acid (C18:1n-9, PUFA) in the case of wild sample. The wild sample gave higher levels of PUFA (with the major contribution of linoleic acid) and MUFA (mainly due to oleic acid), while the commercial sample showed the highest levels of SFA (with the important contribution of palmitic acid).

Although both samples presented similar tocopherol profile (α-, β-, and γ- isoforms), wild yarrow presented higher levels of total tocopherols (**Table 11**), γ-tocopherol being the most abundant isoform. δ-Tocopherol was not found in the samples. Chanishvili et al. 2007 previously reported the presence of tocopherols in *A. millefolium* samples from Georgia, but without quantification of the individual isoforms.

Table 11. Chemical composition of wild and commercial *Achillea millefolium* L. in fatty acids and tocopherols.

	Wild sample	Commercial sample
C6:0	0.72 ± 0.07	0.26 ± 0.03
C8:0	0.05 ± 0.01	0.36 ± 0.04
C10:0	0.20 ± 0.02	4.25 ± 0.37
C11:0	0.05 ± 0.01	0.68 ± 0.01
C12:0	0.09 ± 0.01	0.53 ± 0.06
C13:0	0.02 ± 0.00	0.22 ± 0.02
C14:0	0.05 ± 0.01	1.39 ± 0.12
C14:1	0.03 ± 0.00	0.27 ± 0.09
C15:0	0.07 ± 0.00	0.44 ± 0.02
C15:1	0.09 ± 0.01	0.45 ± 0.04
C16:0	15.54 ± 0.18	20.70 ± 0.17
C16:1	0.06 ± 0.00	1.46 ± 0.06
C17:0	0.26 ± 0.00	0.79 ± 0.02
C18:0	2.85 ± 0.01	6.49 ± 0.07
C18:1n-9	28.23 ± 0.11	9.79 ± 0.00
C18:2n-6	47.16 ± 0.12	26.22 ± 0.10
C18:3n-6	0.10 ± 0.00	3.66 ± 0.03
C18:3n-3	0.23 ± 0.02	11.36 ± 0.70
C20:0	0.72 ± 0.01	1.22 ± 0.04
C20:1	0.30 ± 0.00	0.49 ± 0.03
C20:2	0.08 ± 0.04	0.44 ± 0.32
C20:3n-6	nd	0.20 ± 0.01
C20:4n-6	0.17 ± 0.02	0.46 ± 0.02
C20:3n-3+C21:0	0.47 ± 0.01	0.30 ± 0.00
C20:5n-3	0.96 ± 0.00	0.67 ± 0.17
C22:0	0.79 ± 0.04	2.18 ± 0.15
C22:1n-9	0.04 ± 0.01	0.17 ± 0.15
C23:0	0.14 ± 0.01	0.50 ± 0.02
C24:0	0.55 ± 0.06	4.04 ± 0.06
SFA (g/100 g fat)	22.09 ± 0.22^{b}	44.06 ± 0.74^{a}
MUFA (g/100 g fat)	28.75 ± 0.09^{a}	12.64 ± 0.07^{b}
PUFA (g/100 g fat)	49.16 ± 0.12^{a}	43.30 ± 0.67^{b}
α-tocopherol	0.95 ± 0.21^{a}	0.87 ± 0.14^{a}
β-tocopherol	4.63 ± 0.30^{a}	1.81 ± 0.16^{b}
γ-tocopherol	13.04 ± 1.38^{a}	12.49 ± 1.21^{a}
Total tocopherols (mg/100 g dw)	18.62 ± 1.89^{a}	15.16 ± 1.51^{b}

nd- not detected; dw- dry weight Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Undecylic acid (C11:0); Lauric acid (C12:0); Tridecanoic acid (C13:0); Myristic acid (C14:0); Myristoleic acid (C14:1); Pentadecanoic acid (C15:0); *cis*-10-Pentadecenoic acid (C15:1); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n-9c); Linoleic acid (C18:2n-6c); α-Linolenic acid (C18:3n-3); γ-Linolenic acid (C18:3n-6); Arachidic acid (C20:0); *cis*-11-Eicosenoic acid (C20:1); *cis*-11,14-Eicosadienoic acid (C20:2); Arachidonic acid methyl ester (C20:3n-6); Arachidonic acid methyl ester (C20:4n-6); *cis*-11,14,17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n-3+C21:0); Eicosapentaenoic acid (C20:5n-3); Behenic acid (C22:0); Erucic acid (C22:1n-9); Tricosanoic acid (C23:0); Lignoceric acid (C24:0). SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids. In each row different letters mean significant differences between species ($p<0.05$).

Bioactivity of the methanolic extract, infusion and decoction

Antioxidant properties of the methanolic extract and of the most consumed forms of *A. millefolium*, infusion and decoction, were evaluated and the results are shown in **Table 12**.

In general, commercial yarrow presented lower EC_{50} values (higher antioxidant activity). In both cases (wild and commercial samples), decoctions showed the highest DPPH scavenging activity, β-carotene bleaching inhibition and TBARS inhibition, while infusions presented the highest reducing power.

The samples herein studied gave lower DPPH scavenging activity than water and methanolic extracts of *A. millefolium* from Slovenia and Lithuania (Kintzios et al., 2010; Trumbeckaite et al., 2011). They also showed lower DPPH scavenging activity but higher lipid peroxidation inhibition than methanolic extracts of *A. millefolium* from Turkey (45.60 and 892.67 μg/mL, respectively; Candan et al., 2010). These variations cab be either due to intrinsic factors, mainly genetics or to extrinsic factors, such as storage, type of soil, agronomic practices, climatic factors and technological treatments (Ghasemnezhad, Sherafati, & Payvast, 2011).

The effects of the methanolic extracts, infusions and decoctions on different human tumour cell lines (MCF-7, NCI-H460, HCT-15, HeLa and HepG2) were also evaluated (**Table 12**). The infusion of wild yarrow showed the highest potential against breast (MCF-7; in this case the methanolic extract gave statistically similar results) and hepatocellular (HepG2) carcinoma cell lines, while the methanolic extract of commercial yarrow was most potent against lung (NCI-H460), colon (HCT-15) and cervical (HeLa) carcinoma cell lines. Although the samples present some toxicity for non-tumour liver primary cells (PLP2), the GI_{50} values obtained for tumour cell lines (HepG2) were always lower than the hepatotoxic GI_{50} concentration, suggesting that the samples could be used for antitumour proposes, at the GI_{50} concentration, without toxic effects for non-tumour cells. The results reported for MCF-7 cell line, mainly in the case of decoction and infusion of the commercial sample, are consistent with the ones obtained with ethanolic extracts of *A. millefolium* from Iran (GI_{50}=64.078 μg/mL) (Ghavami et al., 2010). The antiproliferative activity against HeLa and MCF-7 tumour cell lines of sesquiterpene lactones and flavonols isolated from *A. millefolium* samples from Hungary was also studied by Csupor-Löffler et al. (2009) and correlated to the activity of alcoholic and aqueous extracts of the plant.

Table 12. Bioactivity of the methanolic extract, infusion and decoction of wild and commercial *Achillea millefolium* L..

	Wild sample			Commercial sample			Positive control*
	Methanolic extract	***Infusion***	***Decoction***	***Methanolic extract***	***Infusion***	***Decoction***	
Antioxidant activity							
DPPH scavenging activity (EC_{50}, mg/mL)	0.50 ± 0.01^{a}	0.40 ± 0.01^{b}	0.25 ± 0.01^{d}	0.37 ± 0.01^{c}	0.22 ± 0.00^{e}	0.20 ± 0.01^{f}	0.04 ± 0.00
Reducing power (EC_{50}, mg/mL)	0.25 ± 0.01^{b}	0.12 ± 0.00^{e}	0.45 ± 0.00^{a}	0.18 ± 0.01^{d}	0.13 ± 0.00^{e}	0.23 ± 0.00^{c}	0.03 ± 0.00
β-carotene bleaching inhibition (EC_{50}, mg/mL)	2.08 ± 0.04^{a}	0.59 ± 0.30^{b}	0.18 ± 0.03^{c}	0.30 ± 0.21^{c}	0.53 ± 0.06^{b}	0.22 ± 0.00^{c}	0.003 ± 0.00
TBARS inhibition (EC_{50}, mg/mL)	0.81 ± 0.09^{a}	0.45 ± 0.14^{b}	0.04 ± 0.01^{d}	0.26 ± 0.02^{c}	0.07 ± 0.01^{d}	0.08 ± 0.01^{d}	0.004 ± 0.00
Antitumour potential							
MCF-7 (breast carcinoma) (GI_{50}, µg/mL)	17.11 ± 1.05^{c}	14.98 ± 1.68^{c}	64.15 ± 1.75^{a}	48.30 ± 6.07^{b}	64.90 ± 0.79^{a}	64.22 ± 1.02^{a}	0.91 ± 0.04
NCI-H460 (non-small cell lung cancer) (GI_{50}, µg/mL)	54.24 ± 0.46^{a}	29.17 ± 4.12^{b}	56.24 ± 3.09^{a}	24.64 ± 0.80^{b}	56.26 ± 1.15^{a}	55.71 ± 0.04^{a}	1.42 ± 0.00
HCT-15 (colon carcinoma) (GI_{50}, µg/mL)	18.88 ± 0.77^{bc}	15.24 ± 2.10^{c}	22.67 ± 3.82^{ab}	13.90 ± 0.75^{c}	26.23 ± 2.26^{a}	24.27 ± 0.16^{ab}	1.91 ± 0.06
HeLa (cervical carcinoma) (GI_{50}, µg/mL)	39.02 ± 2.90^{b}	20.73 ± 1.16^{c}	52.06 ± 3.87^{a}	19.68 ± 0.47^{c}	47.31 ± 4.84^{ab}	40.96 ± 6.07^{b}	1.14 ± 0.21
HepG2 (hepatocellular carcinoma) (GI_{50}, µg/mL)	47.14 ± 1.85^{b}	37.60 ± 0.86^{b}	61.26 ± 3.77^{a}	41.12 ± 0.54^{b}	67.46 ± 4.47^{a}	66.13 ± 7.10^{a}	3.22 ± 0.67
Hepatotoxicity PLP2 (GI_{50}, µg/mL)	58.14 ± 1.05^{e}	57.08 ± 0.97^{e}	314.41 ± 0.24^{a}	250.42 ± 3.30^{c}	118.95 ± 0.29^{d}	288.82 ± 6.30^{b}	2.06 ± 0.03

*Trolox and ellipticine for antioxidant and antitumour activity assays, respectively. EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p<0.05$).

Phenolic profile of the methanolic extract, infusion and decoction

The HPLC phenolic profile of a wild sample of *A. millefolium* recorded at 280 and 370 nm is shown in **Figure 10**, and peak characteristics and identification are presented in **Table 13**. Twenty-eight compounds were detected, eight of which were phenolic acid derivatives (hydroxycinnamic acid derivatives). Among them, seven compounds (peaks 1, 3, 4, 16, 19, 20 and 22) were caffeoylquinic acid derivatives identified according to their UV spectra and pseudomolecular ions. Peak 1 ($[M-H]^-$ at *m/z* 353) was identified as 3-*O*-caffeoylquinic acid, yielding the base peak at *m/z* 191 and the ion at *m/z* 179 with an intensity >70% base peak, characteristic of 3-acylchlorogenic acids as reported by Clifford, Johnston, Knight, & Kuhnert (2003) and Clifford, Knight, & Kuhnert (2005).

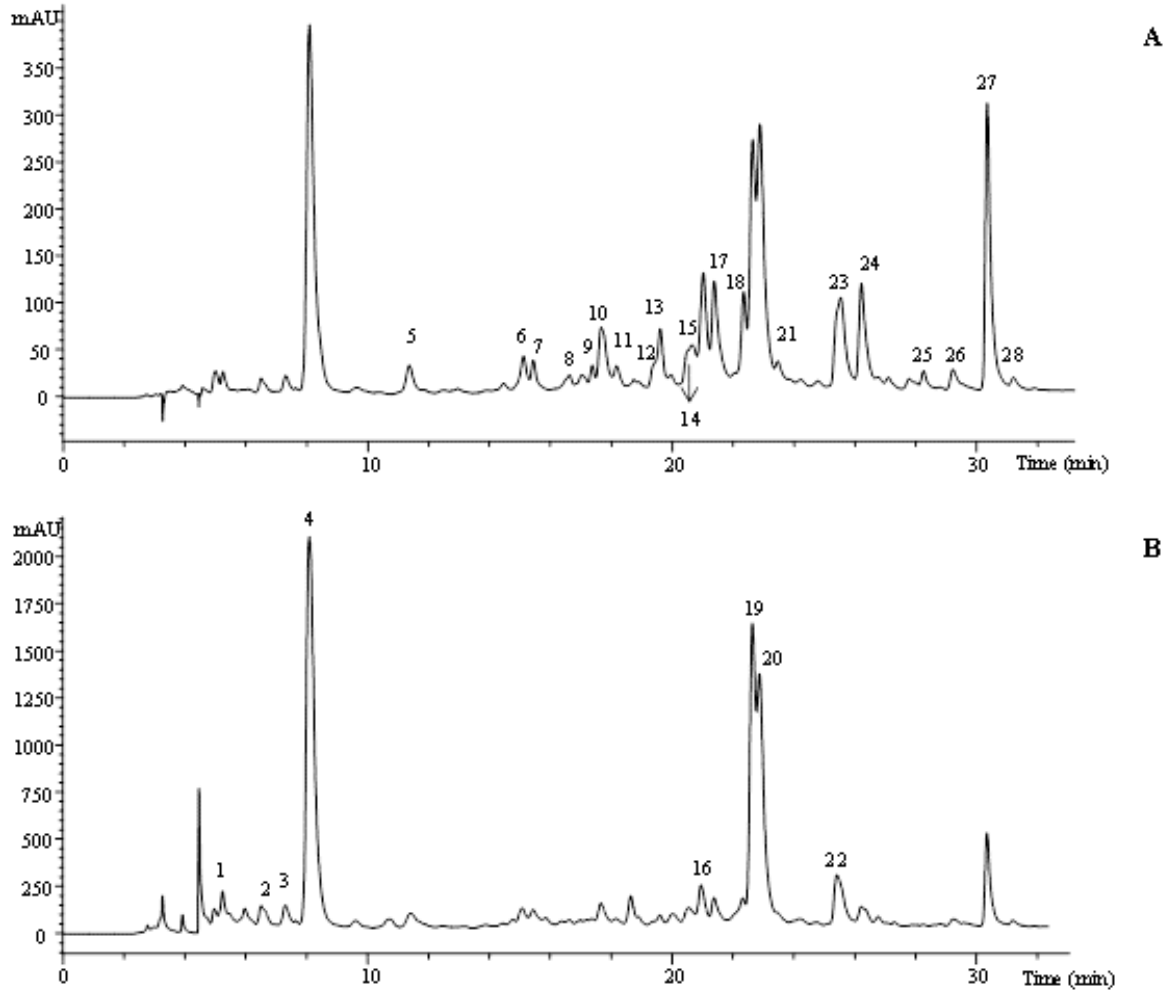

Figure 10. HPLC phenolic profile of wild *Achillea millefolium* L., obtained at 370 nm (A) and 280 nm (B) for flavonoids and phenolic acids, respectively.

Peak 3 was easily distinguished from the other two isomers by its base peak at *m/z* 173 [quinic acid-H-H_2O]$^-$, accompanied by a secondary fragment ion at *m/z* 179 with

approximately 88% abundance of base peak, which allowed identifying it as 4-*O*-caffeoylquinic acid according to the fragmentation pattern described by Clifford et al. (2003, 2005). Peak 4 was identified as 5-*O*-caffeoylquinic acid by comparison of its UV spectrum (λ_{max} 326 nm) and retention time with a commercial standard.

Peaks 16, 19, 20 and 22 ($[M-H]^-$ at *m/z* 515) corresponded to dicaffeoylquinic acids and were assigned to 3,4-*O*-, 3,5-*O*- and 4,5-*O*- dicaffeoylquinic acids, respectively, based on their elution order and fragmentation patterns (Clliford et al., 2003; Clliford et al., 2005). MS^2 fragmentation of peak 16 yielded signals corresponding to "dehydrated" fragment ions at *m/z* 335 [caffeoylquinic acid-H-H_2O]$^-$ and *m/z* 173 [quinic acid-H-H_2O]$^-$, characteristic of 4-acyl-caffeoylquinic acids. Furthermore, according to Clifford et al. (2005), the intensity of signal at *m/z* 335 (34% of base peak), greater than in the other dicaffeoylquinic acids, would allow assigning compound 16 as 3,4-*O*-dicaffeoylquinic acid. The fragmentation pattern of peaks 19 and 20 was similar to the one previously reported by Clifford et al. (2005) for 3,5-*O*-dicaffeoylquinic acid. MS^2 base peak was at *m/z* 353, produced by the loss of one of the caffeoyl moieties [M-H-caffeoyl]$^-$, and subsequent fragmentation of this ion yielded the same fragments as 5-caffeoylquinic acid at *m/z* 191, 179 and 135, although in this case with a comparatively more intense signal at *m/z* 179 [caffeic acid-H]$^-$ (~70% base peak). These peaks 19 and 20 were identified as *cis* and *trans* 3,5-*O*-dicaffeoylquinic acid, respectively, based on the elution order described in a previous study (Barros, Dueñas, Carvalho, Ferreira, & Santos-Buelga, 2012). Compound 22 was assigned to 4,5-*O*-dicaffeoylquinic acid according to its fragmentation, identical to the one previously reported by Clifford et al. (2005). Contrary to 3,4-*O*-dicaffeoylquinic acid (peak 16), in this case the signal at *m/z* 335 was barely detectable (3% of base peak). The intense signal at *m/z* 173, characteristic of an isomer substituted at position 4, would indicate that whereas 3,4-*O*-dicaffeoylquinic acid initially loses the caffeoyl moiety at position 3, the 4,5-*O*-dicaffeoylquinic acid first loses that at position 5. Peak 2 ($[M-H]^-$ at *m/z* 341) was assigned as a caffeic acid hexoside based on the ion at *m/z* 179 (-162 u; hexosyl residue; [caffeic acid-H]$^-$) and UV spectrum (λ_{max} 326 nm).

Flavones were also found in the studied samples, most of them associated to apigenin derivatives (nine compounds) according to their UV spectra (λ_{max} around 330-340 nm) and MS^2 fragmentation pattern (**Table 13**).

Apigenin 7-*O*-glucoside (peak 23) was positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standard. Peaks 5-7 presented pseudomolecular ions $[M-H]^-$ at *m/z* 593 or 563, releasing MS^2 fragment ions corresponding to loss of 90 and 120 mu (*m/z* at 473 and 443), characteristic of *C*-hexosyl flavones, and at *m/z* 383 and 353 that might correspond to the apigenin aglycone plus residues of the sugars that remained linked to it (apigenin + 113 u) and (apigenin + 83 u),

respectively (Ferreres, Silva, Andrade, Seabra, & Ferreira, 2003). The fact that no relevant fragments derived from the loss of complete hexosyl (-162 u) or pentosyl residues (-132 u) were detected suggested that sugars were *C*-attached, which allowed an identification of these compounds as apigenin-*C*-hexoside-*C*-hexoside (Peak 5) and apigenin-*C*-hexoside-*C*-pentoside (peaks 6 and 7).

Peaks 14 and 21 (also pseudomolecular ions at $[M-H]^-$ at *m/z* 593 and 563) could be assigned to an apigenin dihexoside and an apigenin *O*-pentosyl-hexoside, respectively, based on the loss of two hexosyl moieties (162+162 u) in the first case, and of pentosyl and hexosyl residues (132+162 u) in the second one, to yield the aglycone (*m/z* at 269, apigenin). The fact that the two moieties were lost simultaneously suggested that they might constitute a disaccharide *O*-linked to the aglycone.

Peaks 26, 27 and 28, all of them with a pseudomolecular ion $[M-H]^-$ at *m/z* 473 releasing a unique MS^2 fragment at *m/z* 269 (apigenin; $[M-H-42-162]^-$), were identified as apigenin *O*-acetylhexosides according to their mass, 42 u greater than apigenin-hexoside. The observation of three peaks with the same characteristics could be explained by the location of the acetylhexoside moiety on different positions of the aglycone and/or the substitution of the acetyl residue on different positions of the hexose. The positive identification of apigenin 7-*O*-glucoside in the samples would point to one or all of these compounds could be derived from it.

Peaks 8 and 24 were assigned to luteolin derivatives. Peak 8 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 447 giving place to three MS^2 fragment ions, a major one at *m/z* 357 $[M-H-90]^-$, and other two at *m/z* 327 $[M-H-120]^-$ and at *m/z* 297 $[M-H-30]^-$. The fragmentation pattern was characteristic of *C*-glycosylated flavones at C-6/C-8, and the relative abundance of fragments pointed out to sugar substitution at C-6 according to the fragmentation patterns described by Ferreres, Silva, Andrade, Seabra, & Ferreira (2003), Ferreres, Llorach, & Gil-Izquierdo (2004) and Ferreres, Gil- Izquierdo, Andrade, Valentao, & Tomás-Barberán (2007) The peak was identified as luteolin-6-*C*-glucoside, which was further confirmed by comparison to a standard. Peak 24 ($[M-H]^-$ at 489 *m/z*) released a unique MS^2 fragment at *m/z* 285 (luteolin; $[M-H-42-162]^-$) which allowed its identification as luteolin *O*-acetylhexoside.

The remaining phenolic compounds corresponded to flavonols derivatives, most of them derived from quercetin (λ_{max} around 350 nm and an MS^2 fragment at *m/z* 301) (**Table 4**). Quercetin 3-*O*-rutinoside (peak 13) was positively identified according to its retention, mass and UV-vis characteristics by comparison with a commercial standard. Peak 10 ($[M-H]^-$ at *m/z* 463) was assigned to a quercetin hexoside, although the position and nature of the hexosyl moiety could not be identified, because its retention time did not correspond to any

of the standards available (quercetin 3-*O*-glucoside, Rt = 20.05 min.). Peak 9 ($[M-H]^-$ *m/z* at 595) was assigned to a quercetin derivative bearing pentosyl and hexosyl residues, based on the loss of 294 u (132+162 u) to yield the aglycone (*m/z* at 301, quercetin). The fact that the two moieties were lost simultaneously suggested that they could constitute a disaccharide *O*-linked to the aglycone. Peaks 17 and 18 ($[M-H]^-$ at *m/z* 505) should correspond to quercetin *O*-acetylhexosides according to their pseudomolecular ion and MS^2 fragment released at *m/z* 301 (quercetin; $[M-H-42-162]^-$, loss of an acetylhexoside moiety).

Peak 11 ($[M-H]^-$ at *m/z* 695) released a majority MS^2 fragment at *m/z* 651 ($[M-H-44]^-$) interpreted as the loss of CO_2, coherent with the existence of a non-substituted carboxyl. The observation of other fragments at *m/z* 609 ($[M-H-86]^-$) and 447 ($[M-H-86-162]^-$) further support that supposition as they can be interpreted by the loss malonyl and malonylhexosyl residues, respectively. Finally, the fragment at *m/z* 301 ($[M-H-86-162-146])^-$; quercetin) would be explained by further loss of a rhamnosyl residue. The observation of fragments derived from the alternative loss of the malonylhexosyl and the rhamnosyl moieties could suggest that they were located at different positions on the aglycone; however, it might also be rationalised as a quercetin malonylhexosyl-rhamnoside where the two sugars were constituting a disaccharide, in which case the fragment at *m/z* 447 should be explained by structural rearrangement following the loss of the internal malonylhexosyl residue and further linkage of the terminal rhamnose to the aglycone, as observed by (Ma, Li, Van den Heuvel, & Claeys, 1997). In that case, the presence in the samples of quercetin 3-*O*-rutinoside might point to peak 11 as quercetin 3-*O*-malonylrutinoside.

Peak 12 ($[M-H]^-$ at *m/z* 579) was identified as a kaempferol derivative bearing pentosyl and hexosyl residues, owing to the loss of 132+162 u to yield a fragment ion at *m/z* at 285 (kaempferol). The observation that no fragment from the loss of the pentosyl residue was observed pointed to the two sugars were constituting a disaccharide, and the minority fragment ion detected at *m/z* 417 (-162 u, hexosyl residue) suggests that the hexose was the terminal moiety of the disaccharide. Thus, the peak was identified as a kaempferol *O*-pentosyl-hexoside.

Finally, peaks 15 and 25 presented pseudomolecular ions $[M-H]^-$ at *m/z* 477 and 519, which were coherent with an isorhamnetin *O*-hexoside and an isorhamnetin *O*-acetylhexoside, as indicated by the respective losses of 162 u and 162+42 u yielding a unique MS^2 fragment ion at *m/z* 315 (isorhamnetin).

Table 13. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and concentration of phenolic acids and flavonoids in *Achillea millefolium* L.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Identification
1	5.24	326	353	191(100),179(70),173(5),135(53)	3-*O*-Caffeoylquinic acid
2	6.51	326	341	179(100)	Caffeic acid hexoside
3	7.30	328	353	191(50),179(88),173(100),135(70)	4-*O*-Caffeoylquinic acid
4	8.08	326	353	191(100),179(11),173(8),135(5)	5-*O*-Caffeoylquinic acid
5	11.37	330	593	473(19),383(12),353(27)	Apigenin *C*-hexoside-*C*-hexoside
6	15.12	332	563	473(9),443(11),383(20),353(21)	Apigenin *C*-hexoside-C-pentoside
7	15.44	342	563	473(10),443(20),383(15),353(27)	Apigenin *C*-glucose-*C*-pentoside
8	16.36	350	447	357(83),327(88),297(30),285(16)	Luteolin 6-*C*-glucoside
9	17.37	356	595	301(100)	Quercetin *O*-pentosyl-hexoside
10	17.66	344	463	301(100)	Quercetin *O*-hexoside
11	18.17	334	695	651(100),609(3),447(16),301(17)	Quercetin *O*-malonylhexosyl-rhamnoside
12	19.47	350	579	417(7),285(49)	Kaempferol *O*-pentosyl-hexoside
13	19.61	352	609	301(100)	Quercetin 3-*O*-rutinoside
14	20.45	340	593	269(100)	Apigenin *O*-dihexoside
15	20.64	336	477	315(100)	Isorhamnetin *O*-hexoside
16	21.01	328	515	353(71),335(34),299(3),255(4),203(8),191(41),179(70),173(93),161(15),135(32)	3,4-*O*-dicaffeoylquinic acid
17	21.37	346	505	301(100)	Quercetin *O*-acetylhexoside
18	22.35	352	505	301(100)	Quercetin *O*-acetylhexoside
19	22.64	328	515	353(96),335(4),191(100),179(70),173(8),161(14),135(22)	*cis* 3,5-*O*-dicaffeoylquinic acid
20	22.88	330	515	353(96),335(10),191(100),179(68),173(7),161(15),135(15)	*trans* 3,5-*O*-dicaffeoylquinic acid
21	23.46	344	563	269(100)	Apigenin *O*-pentosyl-hexoside
22	25.41	328	515	353(17),335(3),299(5),255(3),203(15),191(49),179(57),173(79),161(14),135(17)	4,5-*O*-dicaffeoylquinic acid
23	25.53	332	431	269(100)	Apigenin 7-*O*-glucoside
24	26.21	350	489	285(100)	Luteolin *O*-acetylhexoside
25	28.25	362	519	315(100)	Isorhamnetin *O*-acetylhexoside
26	29.22	338	473	269(100)	Apigenin *O*-acetylhexoside
27	30.34	336	473	269(100)	Apigenin *O*-acetylhexoside
28	31.20	340	473	269(100)	Apigenin *O*-acetylhexoside

Phenolic acids were the major phenolic compounds present in both wild and commercial samples (**Table 14**), being caffeoylquinic and dicaffeoylquinic acids derivatives the most abundant ones; c*is* and *trans* 3,5-*O*-dicaffeoylquinic acids (peaks 20 and 21) were the compounds found in the highest amounts. Benedek et al. (2007) and Vitalini et al. (2011) also reported 3,5-*O*-dicaffeoylquinic acid as being the main dicaffeoylquinic acid in *A. millefolium* from Austria and Italy, respectively. Those authors also described a similar phenolic profile to the one obtain herein, although with some differences in the flavonoids identified, being apigenin 7-*O*-glucoside, luteolin 7-*O*-glucoside and rutin the main flavonoids reported by them. In our samples luteolin *O*-acetylhexoside and apigenin *O*-acetylhexoside (peaks 24 and 27) were the most abundant flavonoids in both wild and commercial samples. In fact, the presence of acetyl derivatives seems a characteristic of the flavonoid composition in these samples. In this study, besides the mentioned majority flavones, flavonols such as quercetin, kaempferol and isorhamnetin glycosides derivatives were also found, as also *C*-glycosides linkage of apigenin and luteolin, which were not previously reported for this sample. In *A. millefolium* sample from Lithuania, Benetis et al. (2008) described the presence of some similar compounds but they did not identify all the compounds present; the authors identified and quantified only eight phenolic compounds.

Table 14. Phenolic compounds quantification in the methanolic extract (mg/g extract), infusion (mg/g infusion) and decoction (mg/g decoction) of wild and commercial *Achillea millefolium* L..

	Methanolic extract	Infusion	Decoction	Methanolic extract	Infusion	Decoction
Extraction yield (%)	20.39 ± 0.91	21.50 ± 1.02	13.31 ± 0.52	21.32 ± 1.10	22.72 ± 0.48	12.64 ± 0.27
1	0.86 ± 0.04	0.96 ± 0.05	1.22 ± 0.04	0.96 ± 0.07	1.28 ± 0.12	0.89 ± 0.05
2	0.28 ± 0.01	0.21 ± 0.03	0.09 ± 0.01	0.16 ± 0.00	0.21 ± 0.03	0.57 ± 0.03
3	1.01 ± 0.10	1.00 ± 0.00	0.65 ± 0.01	0.31 ± 0.04	0.65 ± 0.04	0.67 ± 0.03
4	24.20 ± 0.18	24.58 ± 0.30	12.76 ± 0.12	13.99 ± 0.64	19.34 ± 0.85	15.24 ± 0.38
5	0.52 ± 0.01	0.76 ± 0.06	0.56 ± 0.05	1.73 ± 0.14	2.28 ± 0.16	2.31 ± 0.10
6	0.75 ± 0.10	0.73 ± 0.02	0.43 ± 0.00	1.18 ± 0.11	1.68 ± 0.13	1.90 ± 0.13
7	0.26 ± 0.02	0.22 ± 0.02	0.17 ± 0.00	0.27 ± 0.01	0.34 ± 0.02	0.42 ± 0.03
8	0.12 ± 0.00	0.12 ± 0.00	0.08 ± 0.00	0.28 ± 0.01	0.32 ± 0.02	0.43 ± 0.05
9	0.15 ± 0.00	0.10 ± 0.00	0.08 ± 0.00	0.35 ± 0.04	0.34 ± 0.01	0.72 ± 0.05
10	2.71 ± 0.12	1.15 ± 0.01	0.29 ± 0.00	0.16 ± 0.04	0.21 ± 0.03	0.18 ± 0.01
11	0.44 ± 0.02	0.19 ± 0.01	0.12 ± 0.01	0.28 ± 0.03	0.22 ± 0.01	0.31 ± 0.03
12	0.29 ± 0.02	0.32 ± 0.01	0.31 ± 0.02	0.49 ± 0.00	0.63 ± 0.04	0.63 ± 0.01
13	0.94 ± 0.03	0.79 ± 0.01	0.10 ± 0.00	0.64 ± 0.02	0.79 ± 0.06	0.86 ± 0.02
14	0.61 ± 0.06	0.98 ± 0.02	0.51 ± 0.06	1.13 ± 0.11	1.52 ± 0.14	1.54 ± 0.05
15	0.34 ± 0.04	0.68 ± 0.04	0.28 ± 0.02	0.29 ± 0.03	0.42 ± 0.07	0.50 ± 0.01
16	5.45 ± 0.19	5.69 ± 0.20	1.60 ± 0.09	4.41 ± 0.27	5.34 ± 0.55	6.16 ± 0.04
17	1.61 ± 0.05	1.88 ± 0.02	0.87 ± 0.06	nd	nd	nd
18	0.76 ± 0.10	0.68 ± 0.09	0.23 ± 0.03	0.55 ± 0.06	0.77 ± 0.08	0.18 ± 0.03
19	35.73 ± 0.44	28.05 ± 0.16	7.40 ± 0.29	25.30 ± 0.24	28.45 ± 2.41	27.83 ± 0.03
20	26.02 ± 0.05	19.96 ± 0.53	6.85 ± 0.05	10.50 ± 0.24	13.46 ± 0.87	11.98 ± 0.28
21	1.13 ± 0.13	1.06 ± 0.01	0.39 ± 0.04	0.88 ± 0.07	0.82 ± 0.32	0.71 ± 0.00
22	10.24 ± 0.02	8.87 ± 0.22	1.94 ± 0.03	10.75 ± 0.67	12.17 ± 0.31	13.53 ± 0.37
23	1.43 ± 0.01	1.20 ± 0.18	0.57 ± 0.00	2.65 ± 0.06	2.56 ± 0.35	2.58 ± 0.10
24	6.21 ± 0.59	5.32 ± 0.05	2.80 ± 0.09	6.49 ± 0.10	7.22 ± 0.04	6.80 ± 0.44
25	0.15 ± 0.01	0.16 ± 0.00	0.24 ± 0.01	0.10 ± 0.00	0.08 ± 0.01	0.07 ± 0.00
26	0.36 ± 0.02	0.35 ± 0.01	0.15 ± 0.01	0.64 ± 0.08	0.75 ± 0.08	0.68 ± 0.03
27	5.45 ± 0.35	5.89 ± 0.04	2.72 ± 0.07	9.85 ± 0.45	12.12 ± 1.04	9.47 ± 0.29
28	0.35 ± 0.00	0.37 ± 0.00	0.25 ± 0.04	0.69 ± 0.06	0.71 ± 0.07	0.79 ± 0.02
TPA	**103.80 ± 0.45^{a}**	**89.32 ± 0.12^{b}**	**32.52 ± 0.52^{e}**	**66.39 ± 2.18^{d}**	**80.91 ± 5.19^{c}**	**76.88 ± 0.39^{c}**
TF	**24.56 ± 0.36^{d}**	**22.96 ± 0.10^{d}**	**11.14 ± 0.05^{e}**	**28.63 ± 1.01^{c}**	**33.78 ± 1.98^{a}**	**31.09 ± 0.47^{b}**
TP	**128.36 ± 0.0^{a}**	**112.28 ± 0.22^{bc}**	**43.66 ± 0.57^{e}**	**95.02 ± 3.19^{d}**	**114.69 ± 7.17^{b}**	**107.97 ± 0.86^{c}**

nd- not detected. TPA- Total phenolic acids; TF- Total flavonoids; TP- Total phenolic compounds. In each row different letters mean significant differences ($p<0.05$).

Regarding contents of total phenolic compounds and phenolic families, different results were obtained depending on the origin of the sample (wild or commercial) and the type of preparation (**Table 14**). Thus, whereas the methanolic extract of wild *A. millefolium* presented higher amount of total phenolic compounds than the commercial sample, the opposite was found in the case of the decoction; infusion yielded more similar amounts of total phenolics in both samples. In all cases phenolic acid derivatives were more abundant than flavonoids, but the contents of these latter were greater in the commercial sample. Benedek et al. (2007) expressed the results in relative percentages, which difficults the comparison with our study; moreover, they reported the presence of 15 compounds whilst 28 are described herein. Vitalini et al. (2011) did not present any type of quantification for

samples of *A. millefolium* from Italy, presenting a profile with 10 different compounds. Benetis et al. (2008) performed the identification and quantification of 8 phenolic compounds, which presented similar values to the ones obtained in our samples.

Overall, commercial yarrow gave higher content of fat (and SFA), proteins, ash, energetic value, total sugars (including fructose, glucose, sucrose and trehalose) and flavonoids (mainly luteolin *O*-acetylhexoside and apigenin *O*-acetylhexoside), while the wild sample revealed higher levels of carbohydrates, organic acids (including malic, oxalic and quinic acids), unsaturated fatty acids, tocopherols (γ-, α- and β-isoforms) and phenolic acids (mainly *cis* and *trans* 3,5-*O*-dicaffeoylquinic acids). In general, commercial yarrow also gave higher antioxidant activity. The decoctions of both samples showed higher free radicals scavenging activity and lipid peroxidation inhibition, while the infusions gave higher reducing power. The methanolic extract of the commercial sample revealed higher antitumour potential against non-small lung, colon and cervical carcinoma cell lines, while the infusion of the wild yarrow gave higher antitumour potential against hepatocellular and breast carcinoma cell lines; for the latter cell line, the methanolic extract showed statistically similar results. The opposite was observed for phenolic compounds concentrations: the methanolic extract of the wild sample revealed the highest levels, while for commercial sample the infusion gave the highest concentration. The heterogeneity among the bioactivity results of the samples and some low correlations with total phenolic acids, flavonoids and phenolic compounds (data not shown) suggested that specific compounds, rather than the totality of them, might be involved in different bioactive properties of samples; the bioactivity could also be related to interactions between specific compounds present in each sample. Moreover, as the most bioactive compounds may be present in lower amounts, further studies should be conducted in order to identify the specific compounds responsible for distinct bioactivities in the samples.

As far as we know, there are no reports of the comparison of different extracts of *A. millefolium*, being this a groundbreaking study on the nutraceutical composition, bioactivity and phenolic profile of wild and commercial yarrow. This study also showed that the chemical qualitative profiles of wild and commercial samples, as also their preparations (i.e., methanolic extract, infusion and decoction) are, in general, similar, varying only in the quantities found. Data obtained are clear evidence that traditional medicinal plants can be used not only in household products but also in pharmaceutical and food industry as a source of new and safer bioactive compounds.

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3.1.1.4. References

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3.2. Fragaria vesca L.

Neste sub-capítulo apresenta-se a caracterização nutricional e química, e as propriedades antioxidantes, citotóxicas, antimicrobianas e inibidoras de biofilme de *Fragaria vesca* L. silvestre e comercial e das respetivas infusões, decocções e extratos metanol: água.

3.2.1. Parâmetros nutricionais das infusões e decocções obtidas a partir de raízes e partes vegetativas de *Fragaria vecsa* L.

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Nutritional parameters of infusions and decoctions obtained from *Fragaria vesca* L. roots and vegetative parts.

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Abstract

Fragaria vesca L. (wild strawberry) roots and vegetative parts are commonly used in infusions and decoctions for different medicinal purposes. The composition in non-nutrients (mainly phenolic compounds) has previously been reported, but the contribution in nutritional compounds has not been researched. Therefore, chemical parameters with nutritional role, namely macronutrients, mineral components, some vitamins (ascorbic acid, folate and tocopherols), as well as, fatty acids, soluble sugars and organic acids, present in *F. vesca* roots and vegetative parts were evaluated using commercial and wild samples. Furthermore, their infusions and decoctions were also fully characterized; as well as the percentages of vitamins and minerals released for the aqueous preparations. The processing steps, the collection region and also the physiological state in which the samples were collected could influence the differences found between commercial and wild samples. The infusion and decoction preparations showed significantly high released percentages of folate and minerals, and also allowed the detection of xylose, proving to be more effective for soluble

sugars extraction. Roots and vegetative parts of *F. vesca*, normally consumed as infusions and decoctions, can be sources of macro and micronutrients.

Keywords: Wild strawberry; Wild/commercial samples; Macronutrients; Minerals; Vitamins

3.2.1.1. Introduction

Fragaria vesca L. (Rosaceae), commonly known as wild strawberry, grows spontaneously in low mountain zones such as forests, slopes and roadsides. It is spread across Europe, being also found in Korea, Japan, North America and Canada (Castroviejo et al., 1998). The leaves of wild strawberry have been traditional used in decoctions against hypertension, presenting also diuretic, antidiarrheal and anticoagulant activity. Decoctions and infusions prepared from the roots are also used to treat urinary tract infections, skin problems, haemorrhoids and cough symptoms (Pawlaczyk, Czerchawski, Pilecki, Lamer-Zarawska & Gancarz, 2009; Camejo-Rodrigues, Ascensão, Bonet & Vallès, 2003; Özüdogru, Akaydın, Erika & Yesila, 2011; Savo, Giulia, Maria & David, 2011). Furthermore, the consumption of roots and vegetative parts (leaves and stems) of *F. vesca* is also believed to increase haematopoiesis, and to have some anti-dysenteric, tonic, antiseptic and detoxifying properties (Neves, Matos, Moutinho, Queiroz & Gomez, 2009; Sõukand & Kalle, 2013).

F. vesca roots and vegetative parts have been reported as sources of non-nutrient compounds, such as procyanidins, ellagic acid and hydroxycinnamic derivatives (Simirgiotis & Schmeda-Hirschmann, 2010; Dias et al., 2014). Nevertheless, to the author's knowledge, there are no reports on nutrients composition of the mentioned parts of *F. vesca,* as well as, their infusions and decoctions. Only the fruits were studied regarding sugars and organic acids (Doumett et al., 2011; Ornelas-Paz et al., 2013), as also the fruits of the hybrid *Fragaria* x *ananassa* Duch. (Hakala, Lapvetelainen, Huopalahti, Kallio & Tahvonen, 2003; Ekholm et al., 2007) concerning minerals content.

A balanced diet containing micronutrients such as vitamins, namely ascorbic acid, folate and tocopherols, and antioxidant compounds is an increasingly central issue for the maintenance of human health and against certain pathologies, such as hypertension and cardiovascular diseases (Houston, 2005). Mineral elements have a very important role in the human health, regarding their physiological functions and requirements. From a nutritional point of view, mineral elements have been classified into two main groups: macroelements, which are needed in higher amounts for physiological function (*e.g.*, potassium, sodium, calcium, magnesium or phosphor), and microelements, in which most of them may be essential to maintain the body functions (*e.g.*, iron, zinc or manganese) (Mahan et al., 2013; Özcan, 2004; Leśniewicz et al., 2006).

The present work intends to improve the knowledge on chemical parameters with nutritional role of *F. vesca* roots and vegetative parts, which have been scarcely studied. Commercial and wild samples were used to prepare infusions and decoctions in order to compare their chemical and nutritional composition with the initial plant matrix, and to determine the percentages of vitamins and minerals released from them to the aqueous preparations (infusions and decoctions).

3.2.1.2. Materials and methods

Standards and Reagents

Acetonitrile (99.9%), n-hexane (95%) and ethyl acetate (99.8%) were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as well as other individual Fatty Acid Methyl Ester isomers, L-ascorbic acid, tocopherol, sugar, organic acid standards, nitric acid and hydrochloric acid. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA). Micro (Fe, Cu, Mn and Zn) and macroelements (Ca, Mg, Na and K) standards (> 99% purity), as well $LaCl_2$ and CsCl (> 99% purity) were purchased from Merck (Darmstadt, Germany). Standars of 5-CH_3-H_4folate monoglutamate (ref. 16252; Schircks laboratories, Jona, Switzerland) and pteroyl diglutamic acid (ref. 16235; Schircks laboratories, Jona, Switzerland), pancreatic chicken homogenate (Pel Freeze, Rogers, Arkansas), rat serum, $NaBH_4$, formaldehyde and octanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile fluorescence grade was bought from Fisher Scientific (Madrid, Spain). All other general laboratory reagents were purchased from Panreac Química S.L.U. (Barcelona, Spain).

Samples and preparation of infusions and decoctions

The commercial samples of *Fragaria vesca* L. vegetative parts and roots were purchased separately in a local supermarket. The wild samples were collected in Serra da Nogueira, Bragança, North-eastern Portugal, in July 2013, and transported to the laboratory in paper bags properly identified. Voucher specimens of the wild samples are deposited in the School of Agriculture Herbarium (BRESA). The vegetative parts and roots were then separated. All the samples were freeze-dried immediately after collection (FreeZone 4.5, Labconco, Kansas, MO, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain homogenate samples.

For infusions preparations, each sample (1 g) was added to 200 mL of boiling distilled water (pH 6.6) at 100ºC and left to stand at room temperature for 5 min; then filtered under reduced pressure (0.22μm). For decoction preparation, each sample (1 g) was added to 200

mL of distilled water (pH 6.6), heated (heating plate, VELP scientific, Keyland Court, NY, USA) and boiled for 5 min at 100^{o}C, in a closed recipient to prevent evaporation. The mixture was left to stand for 5 min and then filtered under reduced pressure (0.22µm). The obtained infusions and decoctions were frozen at -20^{o}C and freeze-dried.

Proximate composition

The samples were analyzed for proteins, fat, carbohydrates and ash according to the AOAC procedures (AOAC, 2005). The crude protein content (N×6.25) was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 550±15^{o}C. Total carbohydrates were calculated by difference.

Minerals composition

Mineral elements analysis was performed according to the method 930.05 of AOAC procedures and following the methodology previously described by the authors (Fernández-Ruiz, Olives, Cámara, Sánchez-Mata & Torija, 2011; Ruiz-Rodríguez et al., 2011). Mineral element analysis was performed on freeze-dried samples. After dry-ash mineralization at 450^{o}C the minerals were extracted in an acid mixture (2 mL HCl 0.5 mL/mL+2 mL HNO_3 0.5 mL/mL) and made up to 50 mL of distilled water. For Ca and Mg determination, a dilution with La_2O_3 (58.6 mg/L deionized water:HCl) was performed in order to avoid interferences. All measurements were performed in atomic absorption spectroscopy (AAS) with air/acetylene flame in Analyst 200 Perkin Elmer equipment (Perkin Elmer, Waltham, MA, USA), comparing absorbance responses with > 99.9% purity analytical standard solutions for AAS made with $Fe(NO_3)_3$, $Cu(NO_3)_2$, Mn $(NO_3)_2$, Zn $(NO_3)_2$, NaCl, KCl, $CaCO_3$ and Mg band. The released percentage of minerals to infusion and decoction preparations was calculated considering the amount of minerals found in the dry samples as 100%.

Soluble sugars

Soluble sugars were determined by high performance liquid chromatography system consisting of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000) and auto-sampler (AS-2057 Jasco, Easton, MD, USA), coupled to a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany), as previously described by the authors (Pereira et al., 2014). The chromatographic separation was achieved with a Eurospher 100-5 NH2 column (5 mm, 250 mm × 4.6 mm i.d., Knauer) operating at 35 ºC (7971 R Grace oven). The mobile phase was acetonitrile (700 mL/L)/deionized water (300 mL/L), at a flow rate of 1 mL/min. The identification was carried out by chromatographic comparisons of the relative retention times

of sample peaks with authentic standards, while the quantification was performed using the internal standard (melezitose) method and by using calibration curves obtained from the commercial standards of each compounds.

The results were expressed in g per 100 g of dry weight for dry plants and in mg per 100 mL for infusion and decoction preparations.

Fatty acids

Fatty acids were determined, after a trans-esterification process as previously described by the authors (Pereira et al., 2014). The fatty acid profile was analysed using a gas-liquid chromatographer (DANI model GC 1000 instrument, Contone, Switzerland) equipped with a split/splitless injector and a flame ionization detection (GC-FID, 260 ºC) and a Macherey–Nagel (Düren, Germany) column (0.5 g/kg cyanopropyl-methyl-0.5 g/kg phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 µm df). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30 ºC/min ramp to 125 ºC, 5 ºC/min ramp to 160 ºC, 20 ºC/ min ramp to 180 ºC, 3 ºC/min ramp to 200 ºC, 20 ºC/min ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (61000 Pa), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC). The identification was made by comparing the relative retention times of FAME (Fatty Acid Methyl Esters) peaks of the samples with commercial standards. The results were recorded and processed using Clarity 4.0.1.7 Software (DataApex, Prague, Czech Republic) and expressed in relative percentage of each fatty acid.

Vitamin C (ascorbic acid) and organic acids

Vitamin C and other organic acids were determined by ultra-fast liquid chromatography coupled to photodiode array detection (UFLC-PDA; Shimadzu Coperation, Kyoto, Japan) and following a procedure previously described by the authors (Pereira et al., 2014). Separation was achieved on a SphereClone (Phenomenex) reverse phase C_{18} column (5 mm, 250 mm × 4.6 mm i.d) thermostatted at 35 ºC. The elution was performed with sulphuric acid 3.6 mmol/L using a flow rate of 0.8 mL/min. The quantification was performed by comparison of the area of the peaks recorded at 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths with calibration curves obtained from commercial standards of each compound: oxalic acid (y=9x10^6x + 377946, R^2=0.994); quinic acid (y =612327x + 16563, R^2=1); malic acid (y =863548x + 55571, R^2=0.999); ascorbic acid (y =10^8x + 751815, R^2=0.998); shikimic acid (y =9x10^7x - 95244, R^2=0.999); citric acid (y =10^6x + 16276, R^2=1); fumaric acid (y =148083x + 96092, R^2=1). The results were expressed in g

per 100 g of dry weight for dry plants and in mg per 100 mL for infusion and decoction preparations.

Folate and tocopherols

Folate content was determined according to the methodology previously described by Morales et al., 2014, using HPLC-FL system, consisted of a Beta 10 (Ecom, Prague, Czech Republic) gradient pump with Gastorr Degasser HPLC Four Channel BR-14 (Triad Scientific, New Jersey, USA) as degassing device, joined to an AS-1555 automatic injector (Jasco, Easton, MD, USA), and to a FP-2020 Plus Fluorescence detector (Jasco, Easton, MD, USA) with RP 18 endcapped Lichrospher 100 column (Merck, Darmstadt, Germany; 250 × 5 mm; 5 µm). The quantification results were obtained from the comparison of the area of the recorded peaks with calibration curves obtained from commercial standards (5-CH_3-H_4folate in both mono and diglutamate forms), and expressed as total folate (from the sum of both compounds). The results were expressed in µg per 100 g of dry weight for dry plants and in µg per 100 mL for infusion and decoction preparations. The released percentage of folate to infusion and decoction preparations was calculated considering the amount of folate found in the dry samples as 100%.

The four isoforms of tocopherols were determined following a procedure previously described by the authors (Pereira et al., 2014), using HPLC coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II normal-phase column (5 mm, 250 mm × 4.6 mm i.d., YMC Waters), operating at 35 °C. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, *v/v*) at a flow rate of 1 mL/min. The identification was performed by chromatographic comparisons with authentic standards, while the quantification was based on the fluorescence signal response of each standard, using the internal standard (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight for dry plants and in mg per 100 mL for infusion and decoction preparations.

Statistical analysis

In each assay, three samples were used and all the analyses were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). Results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 22.0 program (IBM Corp., Armonk, NY, USA).

3.2.1.3. Results and Discussion

Chemical characterization of F. vesca roots and vegetative parts

Results regarding chemical characterization of roots and vegetative parts of *F. vesca* commercial and wild samples are described in **Table 15**. The commercial vegetative parts revealed the highest contents in proteins and fat, while the corresponding wild samples gave the highest ash content.

Table 15. Nutritional value, minerals, soluble sugars, fatty acids, vitamins and organic acids in roots and vegetative parts of *Fragaria vesca* L. commercial and wild samples (mean ± SD; results expressed on dry weight basis).

	Roots		Vegetative parts	
	Commercial	**Wild**	**Commercial**	**Wild**
Nutritional value				
Fat (g/100 g)	1.62 ± 0.01^{c}	1.1 ± 0.1^{d}	2.87 ± 0.05^{a}	2.2 ± 0.1^{b}
Proteins (g/100 g)	3.91 ± 0.01^{b}	4.02 ± 0.02^{b}	6.4 ± 0.5^{a}	2.21 ± 0.08^{c}
Ash (g/100 g)	5.85 ± 0.04^{d}	6.50 ± 0.04^{c}	7.5 ± 0.2^{b}	8.21 ± 0.04^{a}
Carbohydrates (g/100 g)	88.63 ± 0.05^{a}	88.4 ± 0.2^{a}	83.2 ± 0.4^{c}	87.33 ± 0.08^{b}
Microelements (mg/100 g)				
Fe	5.2 ± 0.3^{c}	57 ± 6^{a}	3.8 ± 0.3^{c}	45.3 ± 0.3^{b}
Cu	0.38 ± 0.05^{d}	0.99 ± 0.06^{b}	1.12 ± 0.02^{a}	0.44 ± 0.04^{c}
Mn	0.53 ± 0.04^{d}	14.0 ± 0.8^{b}	7.4 ± 0.8^{c}	18.3 ± 0.8^{a}
Zn	14 ± 1^{a}	8.4 ± 0.3^{b}	4.2 ± 0.3^{c}	3.3 ± 0.1^{d}
Macroelements (mg/100 g)				
Ca	816 ± 27^{c}	929 ± 85^{b}	883 ± 21^{b}	1272 ± 36^{a}
Mg	224 ± 3^{b}	170 ± 5^{d}	230 ± 3^{c}	235.9 ± 0.7^{a}
K	965 ± 17^{b}	192 ± 8^{d}	1700 ± 28^{a}	674 ± 13^{c}
Soluble sugars (g/100 g)				
Fructose	4.2 ± 0.3^{a}	2.08 ± 0.06^{b}	1.7 ± 0.2^{c}	1.63 ± 0.04^{c}
Glucose	4.0 ± 0.2^{a}	2.44 ± 0.03^{c}	3.76 ± 0.08^{b}	1.71 ± 0.09^{d}
Sucrose	0.20 ± 0.02^{d}	13.5 ± 0.1^{a}	0.40 ± 0.01^{c}	1.76 ± 0.08^{b}
Trehalose	0.23 ± 0.01^{d}	2.62 ± 0.08^{a}	0.5 ± 0.1^{c}	0.69 ± 0.02^{b}
Raffinose	nd	nd	nd	0.29 ± 0.03
Sum	8.7 ± 0.5^{b}	20.66 ± 0.06^{a}	6.4 ± 0.2^{c}	6.08 ± 0.03^{c}
Fatty acids (relative percentage)				
C16:0	26.9 ± 0.4	15.8 ± 0.2	21.6 ± 0.8	16 ± 2
C18:0	8.91 ± 0.04	3.9 ± 0.1	6.41 ± 0.04	5.3 ± 0.6
C18:1n9	10.5 ± 0.1	7.9 ± 0.2	8.0 ± 0.4	5.1 ± 0.3
C18:2n6	31.0 ± 0.1	45.2 ± 0.2	18.06 ± 0.04	7.8 ± 0.2
C18:3n3	11.4 ± 0.5	15.32 ± 0.08	21.4 ± 0.3	24.8 ± 0.7
C20:0	2.33 ± 0.01	2.5 ± 0.2	3.7 ± 0.3	7.7 ± 0.6
C20:5n3	nd	nd	3.4 ± 0.9	8 ± 2
C22:0	2.01 ± 0.03	2.8 ± 0.2	4.4 ± 0.5	9 ± 1
C24:0	1.35 ± 0.03	2.69 ± 0.04	3.6 ± 0.5	8 ± 1
SFA	45.9 ± 0.6^{b}	30.7 ± 0.4^{c}	45.6 ± 0.2^{b}	53 ± 3^{a}
MUFA	11.38 ± 0.03^{a}	8.3 ± 0.1^{c}	10.5 ± 0.5^{b}	5.6 ± 0.2^{d}
PUFA	42.7 ± 0.6^{c}	60.9 ± 0.3^{a}	43.9 ± 0.3^{b}	41 ± 3^{d}
Vitamin C (Ascorbic acid, mg/100 mg)	nd	tr	nd	tr
Vitamin B_9 (Folate, µg/100 g)	149 ± 3^{b}	253 ± 20^{a}	62.6 ± 0.3^{d}	115 ± 3^{c}
Vitamin E (Tocopherols, mg/100 g)				
α-Tocopherol	1.36 ± 0.01^{d}	65.00 ± 0.01^{a}	2.9 ± 0.3^{c}	3.3 ± 0.3^{b}
β-Tocopherol	nd	1.61 ± 0.01^{a}	nd	0.38 ± 0.04^{b}
γ-Tocopherol	0.15 ± 0.01^{d}	2.52 ± 0.01^{a}	0.29 ± 0.01^{c}	1.0 ± 0.1^{b}

δ-Tocopherol	nd	2.42 ± 0.01[a]	nd	1.3 ± 0.2[b]
Sum	1.50 ± 0.02[d]	71.56 ± 0.01[a]	3.2 ± 0.3[c]	6.5 ± 0.6[b]
Organic acids (g/100 g)				
Oxalic acid	1.26 ± 0.03[a]	0.26 ± 0.01[c]	0.59 ± 0.01[b]	0.26 ± 0.04[c]
Quinic acid	nd	nd	0.85 ± 0.17[a]	0.24 ± 0.02[b]
Malic acid	2.1 ± 0.3[a]	tr	1.13 ± 0.16[b]	0.54 ± 0.07[c]
Shikimic acid	0.01 ± 0.00[b]	nd	0.04 ± 0.00[a]	nd
Citric acid	nd	nd	2.86 ± 0.07[b]	3.44 ± 0.16[a]
Fumaric acid	0.002 ± 0.00[b]	nd	0.01 ± 0.00[a]	nd
Sum	3.4 ± 0.3[c]	0.26 ± 0.01[d]	5.48 ± 0.07[a]	4.5 ± 0.3[b]

nd- not detected; tr- traces; Fe- iron Cu- cooper, Mn- manganese, Zn- zinc, Ca- calcium, Mg- magnesium, K- potassium; C16:0- palmitic acid, C18:0- stearic acid, C18:1n9- oleic acid, C18:2n6- linoleic acid, C18:3n3- linolenic acid, C20:0- arachidic acid, C20:5n3- cis-5,8,11,14,17-eicosapentaenoic acid, C22:0- behenic acid, C24:0- lignoceric acid; SFA- saturated fatty acids, MUFA- monounsaturated fatty acids, PUFA- polyunsaturated fatty acids. In each row different letters mean significant differences between samples (p□0.05), where “a”and “d” correspond to the highest and lowest values, respectively.

Regarding minerals composition, the wild roots and vegetative parts gave very high amount of iron and manganese microelements, while commercial vegetative parts and wild roots gave the highest amount of copper and zinc, respectively. In terms of macroelements, the highest levels of calcium and magnesium were found in wild vegetative parts, while the highest potassium concentration was observed in commercial vegetative parts.

The soluble sugars detected in the four studied samples presented some similarities; raffinose was only detected in wild vegetative parts (**Table 15**). The highest total soluble sugars content was observed in wild roots sample (20.66 g/100 g), mainly due to the presence of sucrose (13.53 g/100 g), which was also present in high concentration in the wild vegetative parts of *F. vesca* (1.76 g/100 g). Commercial roots and vegetative parts samples presented fructose and glucose as the major ones, followed by sucrose and trehalose.

Regarding fatty acids profile, 22 compounds were identified; the most abundant ones in the four studied samples are presented in **Table 15**. Linoleic acid (C18:2n6) was the major fatty acid found in commercial and wild roots samples (30.97 and 45.16%, respectively) followed by palmitic acid (C16:0; 26.93 and 15.82%, respectively). Contrarily, in commercial vegetative parts, palmitic acid (C16:0) was the major acid, while linoleic acid (C18:3n3) was the most abundant in wild vegetative parts. Eicosapentaenoic acid (C20:5n3) was not detected in root samples. The highest concentration of polyunsaturated fatty acids (PUFA; 60.91%) was observed in the wild roots sample. Saturated fatty acids (SFA) are also present in high concentrations followed by monounsaturated fatty acids (MUFA) in all samples.

Folate was found in higher amounts in wild roots sample (253.3 μg/100 g), followed by commercial roots and wild and commercial vegetative parts. Regarding tocopherols content, the wild roots sample also presented the highest concentration mainly due to α-tocopherol (65 mg/100 g). Commercial roots and vegetative parts samples showed only the presence of α- and γ-tocopherols. Both vitamins are highly degradable molecules and,

therefore, these results can be explained by the less processing steps to which wild samples were submitted: freeze drying immediately after collection, which preserves ascorbic acid by means of freezing temperatures and oxygen absence (Davey et al., 2000); some authors proved the effects of storage and freeze drying effects in the stability of folate proving that blanching lead to a decrease of half of the folate content on vegetables (Puupponen-Pimia et al., 2003); and also the effects of temperature on tocopherols content in vegetables, seeing that cooking and baking process lead to a decrease on tocopherol availability (Knecht et al., 2015). Only trace amounts of ascorbic acid in wild root sample, this may be explained by the fact that this molecule competes directly by the oxygen present in the sample and processing steps may also have led to its degradation. (Allwood & Martin, 2000).

The organic acids profile varied depending on the plant material analysed; these compounds are normally found in higher amounts in aerial parts, where their biosynthesis is increased. Furthermore, its content is highly influenced by the environmental conditions (López-Bucio et al., 2003). As expected, organic acids profile was very different between samples, due to the different plant material analysed. Vegetative parts revealed the presence of more organic acids, revealing commercial sample the highest amount (5.48 g/100 g). Wild roots presented only oxalic acid, while commercial roots gave malic acid as the major organic acid.

Chemical and nutritional characterization of infusions and decoctions prepared from F. vesca roots and vegetative parts

The results of chemical and nutritional characterization in infusions and decoctions prepared from roots of *F. vesca* commercial and wild samples are provided in **Table 16**. In general, micro and macroelements were found in higher amounts in the infusions. Iron and zinc were more abundant in commercial roots infusion (0.04 and 0.08 mg/100 mL), while cooper and manganese predominated in wild roots infusion (0.03 and 0.06 mg/100 mL); copper was not detected in wild roots decoction sample. Calcium, magnesium and potassium were found in commercial roots infusion in the highest concentrations (5.82, 3.48 and 4.12 mg/100 mL, respectively).

Table 16. Minerals, soluble sugars, vitamins and organic acids in infusions and decoctions prepared from roots of *Fragaria vesca* L. commercial and wild samples (mean ± SD).

	Commercial Roots		Wild Roots	
	Infusion	**Decoction**	**Infusion**	**Decoction**
Ash content (g/100 mL)	0.04 ± 0.01^{d}	0.38 ± 0.06^{a}	0.14 ± 0.02^{b}	0.11 ± 0.02^{c}
Microelements (µg/100 mL)				
Fe	40 ± 1^{a}	20 ± 1^{b}	20 ± 1^{b}	20 ± 1^{b}
Cu	10 ± 1^{b}	2.0 ± 0.5^{c}	30 ± 1^{a}	nd
Mn	2.0 ± 0.5^{c}	4.0 ± 0.5^{c}	60 ± 1^{a}	30 ± 1^{b}
Zn	80 ± 1^{a}	60 ± 1^{b}	20 ± 1^{c}	10 ± 1^{d}
Macroelements (mg/100 mL)				
Ca	5.8 ± 0.6^{a}	5.3 ± 0.3^{b}	3.65 ± 0.06^{c}	3.24 ± 0.06^{c}
Mg	3.5 ± 0.4^{a}	3.2 ± 0.1^{b}	1.52 ± 0.01^{c}	0.72 ± 0.02^{d}
K	4.12 ± 0.09^{a}	2.43 ± 0.06^{b}	0.27 ± 0.01^{d}	1.4 ± 0.1^{c}
Soluble sugars (mg/100 mL)				
Xylose	0.59 ± 0.08^{b}	0.58 ± 0.06^{b}	0.34 ± 0.07^{c}	0.88 ± 0.05^{a}
Fructose	18.0 ± 0.3^{a}	17.77 ± 0.17^{b}	1.66 ± 0.13^{d}	4.58 ± 0.09^{c}
Glucose	13.6 ± 0.1^{a}	14.18 ± 0.02^{b}	1.53 ± 0.17^{d}	4.25 ± 0.03^{c}
Sucrose	2.3 ± 0.4^{c}	2.75 ± 0.00^{b}	1.81 ± 0.25^{d}	3.75 ± 0.09^{a}
Trehalose	1.3 ± 0.2^{a}	1.22 ± 0.06^{b}	0.25 ±0.02^{d}	0.80 ± 0.05^{c}
Sum	36.0 ± 0.9^{a}	36.5 ± 0.3^{a}	5.6 ± 0.6^{c}	14.3 ± 0.2^{b}
Vitamin C (Ascorbic acid, mg/100 mL)	nd	nd	nd	nd
Vitamin B_9 (Folate, µg/100 mL)	10 ± 1^{c}	10.6 ± 0.1^{d}	28.1 ± 0.7^{a}	26 ± 3^{b}
α-Tocopherol (µg/100 mL)	0.32 ± 0.01^{a}	0.20 ± 0.03^{b}	0.04 ± 0.01^{c}	0.19 ± 0.01^{b}
Organic acids (mg/100 mL)				
Oxalic acid	4.15 ± 0.05^{b}	4.48 ± 0.04^{a}	0.25 ± 0.01^{d}	1.35 ± 0.06^{c}
Malic acid	5.4 ± 0.4^{a}	4.9 ± 0.8^{b}	tr	tr
Shikimic acid	0.06 ± 0.01^{a}	0.05 ± 0.01^{a}	nd	nd
Fumaric acid	tr	tr	nd	nd
Sum	9.6 ± 0.4^{a}	9.5 ± 0.8^{a}	0.25 ± 0.01^{c}	1.35 ± 0.06^{b}

nd- not detected; tr- traces; Fe- iron Cu- cooper, Mn- manganese, Zn- zinc, Ca- calcium, Mg- magnesium, K- potassium. In each row different letters mean significant differences between samples (p>0.05), where “a”and “d” correspond to the highest and lowest values, respectively.

The soluble sugars profile is very similar among all the samples; the highest sum was found in commercial roots infusions and decoctions samples (35.97 and 36.51 mg/100 mL, respetively), mainly due to the presence of high concentrations of glucose and fructose. For wild roots samples, the decoction presented the highest level of sugars (14.26 mg/100 mL), being also found xylose.

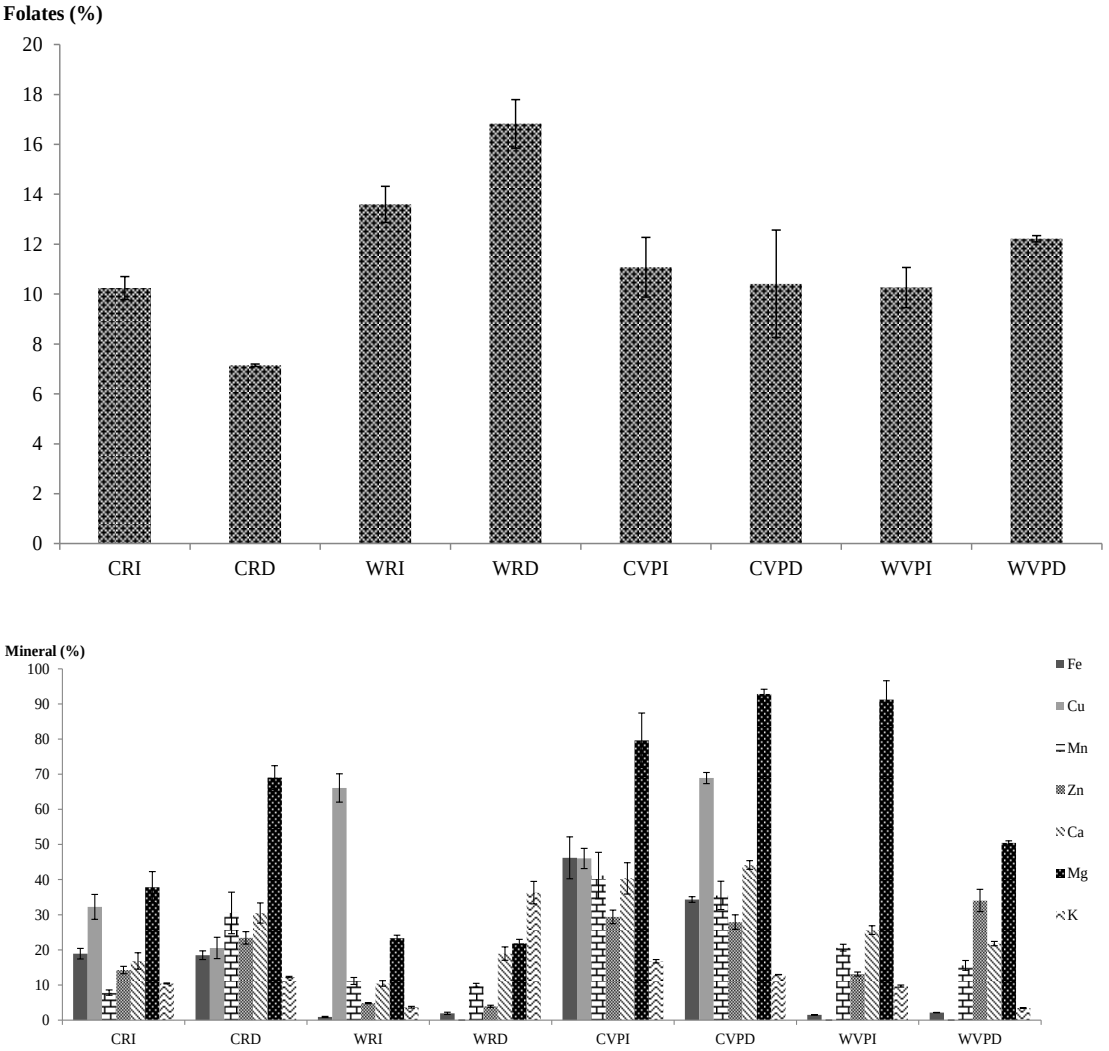
Folate content was higher in wild roots decoction and infusion sample (26.37 and 28.06 µg/100 mL, respectively), while α-tocopherol was the only isoform of tocopherols identified in all the analysed samples, presenting commercial roots infusion the highest amount (0.32 µg/100 mL). The level of ascorbic acid present in the plant samples was very low (traces amounts), which might explain the fact of not being detected in the infusions and decoctions. Besides, it is known that this compound decreases with increasing temperature (Lester, 2006).

Organic acids were also present in higher amounts in the commercial roots samples, mainly due to the contribution of malic acid (infusion and decoction, 5.37 and 4.93 mg/100 mL, respectively). The profiles were very different in the studied samples; oxalic and malic acids were only identified in the wild samples infusion and decoction, being the last one presented in traces amount; this was also observed in the wild root sample, while commercial roots, commercial vegetative parts and wild vegetative parts samples presented it as the second major compound.

Regarding *F. vesca* commercial and wild vegetative part samples (**Table 17**), the infusion of commercial vegetative parts presented the highest levels of macro and microelements; copper was not detected in the wild samples infusion and decoction. Similarly to root samples, it is in the commercial vegetative parts samples (infusion and decoction) that sugars and organic acids were found in the highest amounts. In the case of sugars, fructose and glucose were once more found in the highest concentrations in commercial vegetative part infusions and decoctions (40.44 and 39.86 mg/100 mL, respectively); xylose was also found in the infusions and decoctions of vegetative parts. The presence of xylose on water extracts can be explained by the more extractability capacity of infusions and decoctions, existing in a free form but not being detected in the dry samples (less extractability capacity).

In terms of organic acids, the highest amounts were found in commercial vegetative parts infusions and decoctions (58.79 and 68.0 mg/100 mL, respectively), mainly due to citric acid, which is in accordance with the content found in the vegetative parts (**Table 15**); shikimic and fumaric acids were not detected in the wild samples, while fumaric acid was only detected in traces amount in commercial samples. In the decoctions of wild vegetative parts, higher amounts of folate (13.99 µg/100 mL) and α-tocopherol (0.33 µg/100 mL) were found; different results were obtained for root samples.

As mentioned before, some highly thermal sensible vitamins, as folate and tocopherols (Puupponen-Pimia et al., 2003; Knecht et al., 2015), were characterized in decoctions and infusions of *F. vesca* samples. Furthermore, their release percentage from plant matrix was calculated and showed in **Figure 11A**. The highest folate release percentage was found in commercial vegetative part infusions and decoctions (13.59% and 16.82%, respectively) and in wild vegetative part decoctions (12.22%). Moreover, after thermal treatment the release percentage of tocopherols was also higher in the infusions than in decoctions but in all cases, lower than 2% (data not shown), mainly due to the lipophilic character of vitamin E.



nd- not detected; tr- traces; Fe- iron Cu- cooper, Mn- manganese, Zn- zinc, Ca- calcium, Mg- magnesium, K- potassium. In each row different letters mean significant differences between samples ($p<0.05$), where “a”and “d” correspond to the highest and lowest values, respectively.

Figure 11. Folates (**A**) and minerals (**B**) release percentage after infusions and decoctions preparation from roots and vegetative parts of commercial and wild *Fragaria vesca* L. samples.

Table 17. Minerals, soluble sugars, vitamins and organic acids in infusions and decoctions prepared from vegetative parts of *Fragaria vesca* L. commercial and wild samples (mean ± SD).

	Commercial vegetative parts		Wild vegetative parts	
	Infusion	**Decoction**	**Infusion**	**Decoction**
Ash content (g/100 mL)	0.24 ± 0.03^{b}	0.24 ± 0.01^{b}	0.24 ± 0.03^{b}	0.37 ± 0.04^{a}
Microelements (µg/100 mL)				
Fe	70 ± 1^{a}	30 ± 1^{b}	10 ± 1^{d}	20 ± 1^{c}
Cu	20 ± 1	20 ± 1	nd	nd
Mn	130 ± 1^{a}	60 ± 1^{c}	70 ± 1^{b}	60 ± 1^{c}
Zn	50 ± 1^{a}	20 ± 1^{b}	10 ± 1^{c}	20 ± 1^{b}
Macroelements (mg/100 mL)				
Ca	14 ± 2^{a}	8.47 ± 0.06^{b}	6.5 ± 0.2^{c}	5.29 ± 0.01^{d}
Mg	7.3 ± 0.7^{a}	4.65 ± 0.01^{b}	4.2 ± 0.2^{b}	2.32 ± 0.02^{c}
K	11.4 ± 0.1^{a}	4.79 ± 0.07^{b}	1.26 ± 0.03^{c}	0.46 ± 0.01^{d}
Soluble sugars (mg/100 mL)				
Xylose	2.1 ± 0.1^{c}	1.76 ± 0.03^{d}	5.82 ± 0.07^{a}	3.31 ± 0.03^{b}
Fructose	11.7 ± 0.2^{a}	11.7 ± 0.6^{a}	6.4 ± 0.1^{b}	4.19 ± 0.09^{c}
Glucose	16.29 ± 0.09^{b}	17.7 ± 0.6^{a}	7.42 ± 0.01^{c}	4.8 ± 0.2^{d}
Sucrose	7.1 ± 0.3^{b}	6.0 ± 0.2^{c}	8.53 ± 0.05^{a}	2.91 ± 0.03^{d}
Trehalose	3.2 ± 0.3^{b}	2.7 ± 0.3^{c}	3.56 ± 0.02^{a}	1.77 ± 0.06^{d}
Sum	40.4 ± 0.4^{a}	39.9 ± 0.5^{b}	31.7 ± 0.2^{c}	17.0 ± 0.2^{d}
Vitamin C (Ascorbic acid, mg/100 mL)	nd	nd	nd	nd
Vitamin B_9 (Folate, µg/100 mL)	8.5 ± 0.5^{b}	10.5 ± 0.6^{d}	11.7 ± 0.7^{c}	13.9 ± 0.2^{a}
α-Tocopherol (µg/100 mL)	0.10 ± 0.01^{d}	0.33 ± 0.02^{a}	0.22 ± 0.01^{b}	0.20 ± 0.01^{c}
Organic acids (mg/100 mL)				
Oxalic acid	1.18 ± 0.09^{b}	2.4 ± 0.5^{a}	2.51 ± 0.01^{a}	0.74 ± 0.07^{c}
Quinic acid	1.2 ± 0.2^{c}	1.5 ± 0.1^{b}	4.56 ± 0.08^{a}	4.5 ± 0.1^{a}
Malic acid	2.0 ± 0.1^{c}	2.8 ± 0.4^{b}	1.8 ± 0.1^{c}	27.2 ± 0.5^{a}
Shikimic acid	0.13 ± 0.01^{b}	0.20 ± 0.01^{a}	nd	nd
Citric acid	54 ± 6^{b}	61 ± 4^{a}	1.08 ± 0.06^{c}	0.56 ± 0.07^{c}
Fumaric acid	tr	tr	nd	nd
Sum	59 ± 6^{b}	68 ± 3^{a}	9.99 ± 0.03^{d}	33.1 ± 0.2^{c}

Infusion and decoction minerals release percentage was calculated, being illustrated in **Figure 11B**. The vegetative parts of *F. vesca* provided higher deliver percentages of micro and macroelements to the infusions and decoctions. Copper (with the exception to wild vegetative parts infusions and decoctions, in which copper was not detected) and magnesium represented the micro and macroelements with the highest released percentages for the infusions and decoctions. The maximal released percentage for copper observed in commercial vegetative parts decoction sample (~69%), while for magnesium was observed in wild vegetative parts infusion (~91%). The commercial vegetative parts infusion sample presented also the highest released percentage for iron (~46%), manganese (~41%), zinc (~29%) and calcium (40%). Otherwise, potassium reached the maximal released percentage in wild roots decoction sample (~36%).

In general, the amount of each nutrient found in the infusion or decoction liquid, would be the result of the balance between extraction rate, and non-diffusion to water. Both are expected to be higher in decoctions, where boiling temperatures are maintained during 5

min, with respect to infusions where temperature decreases during this time. As a result, lipophilic compounds (such as tocopherols) are not expected to be extracted in a high extent into the liquid (aqueous environment) being also highly prone to thermal degradation; hydrophilic substances would behave in a different way depending on their thermal stability: mineral elements, highly stable, are in many cases more extracted into decoction liquids (higher exposition time at boiling temperature), while folate could suffer some degradation in these conditions.

Iron, manganese, zinc and calcium also showed lower released percentages when compared to the results obtained for our samples. Herbal infusion mixtures containing several plants were also studied for their content in macro and microelements in comparison with the dry plant; the authors obtained good results in the amount of minerals that are released to the infusion, however, unlike the herein observed, Mn was the more soluble component. In the present study, Cu, Zn and Na were the elements released in the highest amounts to the infusions (Aldars-García, Zapata-Revilla & Tenorio-Sanz, 2013). Łozak, Sołtyk, Ostapczuk & Fijałek (2002) also studied the percentage of released minerals from plant to infusions of *Menthae piperitae folium*. (mint) and *Urticae folium* (nettle), describing much lower values for Mg (38 and 25% for mint and nettle, respectively) and Cu (25 and 33% for mint and nettle, respectively) in comparison with the herein studied sample commercial vegetative parts decoction.

Overall, fruits are the most commonly studied part of *F. vesca*. However, and despite the various ethnobotanical uses reported for vegetative parts and roots, their nutritional characterization has been discarded. The present study proved that *F. vesca* roots and vegetative parts (either commercial or wild samples) are sources of nutrients and molecules with high physiological and nutritional importance, such as tocopherols (α-tocopherol), folate, mineral elements, soluble sugars and organic acids. Moreover, according to the regulation of the European Parliament the reference daily intake (RDA) of folate is 200 μg/day (Regulation (EC) No 1169/2011), and some of the studied samples (wild roots) presented a release of folate to infusions and decoctions higher than 14% towards providing this RDA.

Even though some nutrients losses were observed during infusions and decoctions preparation, the release percentages of folate and minerals in the aqueous extracts are significantly high. Tocopherols almost disappear after infusion and decoction elaboration, which was expectable due to their lipophilic properties and its low thermal stability. Infusion and decoction preparations proved to be also effective for soluble sugars extraction allowing the detection of xylose.

The qualitative differences found in some chemical profiles of commercial and wild samples can be explained by several factors such as the processing steps, the collection region, as also the physiological state of the samples (Tiwari & Cummins, 2013).

The present work shows the huge potential of roots and vegetative parts of *F. vesca*, normally consumed as infusions and decoctions, in order to provide different macro and micronutrients.

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3.2.1.4. References

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3.2.2. Perfil fenólico e propriedades antioxidantes de raízes comerciais e silvestres de *Fragaria vesca* L.: comparação entre extratos metanol: água e aquosos

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Phenolic profile and antioxidant properties of commercial and wild *Fragaria vesca* L. roots: A comparison between hydromethanolic and aqueous extracts

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Running title: Phenolic profile and antioxidant properties of *Fragaria vesca* L. roots

Abstract

The phenolic profile of hydromethanolic extracts, infusions and decoctions of commercial and wild samples of *Fragaria vesca* (wild strawberry) roots was obtained by HPLC-DAD/ESI-MS, and further correlated with their antioxidant properties. Commercial and wild samples showed similarities in terms of flavan-3-ols (TF3O), with catechin derivatives, mainly procyanidins, as major compounds in both samples. The commercial sample presented ellagic acid glycosides, whereas the wild sample presented flavonols (TF) and dihydroflavonols (TdhF, taxifolin derivatives). The infusion of wild sample gave the highest content of total phenolic compounds (TPC), DPPH (2,2-Diphenyl-1-picrylhydrazyl) scavenging activity, reducing power and TBARS (thiobarbituric acid reactive substances) inhibition. The antioxidant capacity (mainly β-carotene bleaching and TBARS inhibition)

observed for the wild sample is correlated with TF3O, TF and TPC. Overall, the high antioxidant potential of *F. vesca* roots was demonstrated and could be achieved directly by consumption of infusions/decoctions or by incorporating hydromethanolic extracts in antioxidant formulations.

Keywords: Fragaria vesca L.; commercial/wild; alcoholic/aqueous extracts; phenolic compounds; antioxidant activity.

3.2.2.1. Introduction

With the increasing aging of the world's population and simultaneously the lifestyle that society has today, the occurrence of oxidative stress in cells, and consequently, the production of reactive species of oxygen (ROS) is also increasing, which has been related with a higher incidence of cardiovascular, brain and immune system diseases (Carocho and Ferreira, 2013). To prevent, delay or stop this process, antioxidants obtained from herbs may act as reducing agents, free radical scavengers or singlet oxygen quenchers. Through synergistic and additive effects of those bioactive compounds, natural extracts can provide higher beneficial effects when compared to individual molecules (Liu, 2003).

Fragaria vesca L., wild strawberry, belongs to Rosaceae family and is commonly found in forests, slopes and roadsides. Widely spread across Europe, it can also be found in Korea, Japan, North America and Canada (Castroviejo et al., 1998). The roots of wild strawberry are traditionally used to prepare decoctions and infusions for cough symptoms, urinary tract infections, haemorrhoids, diarrhoea, and gout. These preparations also show diuretic properties, anti-dysenteric and antiseptic capacity, functioning as detoxifier, emollient and dermatologic protector (Camejo-Rodrigues et al., 2003; Neves et al., 2009; Özüdogru et al., 2011; Savo et al., 2011).

The bioactive properties related to the fruits, leaves and also roots of strawberry are mainly due to the composition in phenolic compounds, including anthocyanins, proanthocyanidins, flavonols, and derivatives of hydroxycinnamic and ellagic acids (Simirgiotis and Schmeda-Hirschmann, 2010; Sun et al., 2014). Ellagic acid, one of the bases of hydrolysable tannins, is very interesting because it can mostly be found in some berries and nuts. Normally, it is present as ellagitannins or esterified with glucose, while the free form of this compound is rarely found (Clifford and Scalbert, 2000; Pinto et al., 2008). Proanthocyanidins, condensed tannins, can be also found in high concentrations in berries, although they are usually underestimated due to the difficulties associated with extraction, separation and analysis methodologies (Aaby et al., 2012).

There are many reports on the phenolic compounds of *Fragaria x ananassa* variety (Aaby et al., 2012; Andersen et al., 2004; Bodelón et al., 2010; Bordonaba et al., 2011;

Fossen et al., 2004; Holzwarth et al., 2012; Lopes da Silva et al., 2007; Pinto et al., 2008; Tarola et al., 2013; Theocharis and Andlauer, 2013), but only a few studies are available regarding phenolic composition of *F. vesca* fruits (Bubba et al., 2012; Gasperotti et al., 2013; Sun et al., 2014; Zheng et al., 2007).

The antioxidant properties of *F. vesca* fruits, leaves (Nuñez-Mancilla et al., 2013; Raudonis et al., 2012), pulp (Özşen and Erge, 2013), achenes and thalamus (Cheel et al., 2007), and of fruits, leaves and roots of *F. chiloensis* (Simirgiotis and Schmeda-Hirschmann, 2010) were also described. However, as far as we know, there are no reports on the phenolic profile and antioxidant activity of *F. vesca* roots. Therefore, in the present study, commercial and wild samples of this material were submitted to different extraction procedures in order to compare their antioxidant potential. Infusions and decoctions were prepared due to their common consumption, while hydromethanolic extracts (the most common procedure to obtain phenolic compounds enriched extracts) could be incorporated in bioactive formulations.

3.2.2.2. Materials and methods

Samples

The commercial samples of *Fragaria vesca* L. roots were purchased in a local supermarket, while the wild samples were collected in Serra da Nogueira, Bragança, North-eastern Portugal, in July 2013. Voucher specimens (nº 9687) are deposited in the School of Agriculture Herbarium (BRESA). All the samples were lyophilized (FreeZone 4.5, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain homogenate samples.

Standards and Reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic acid was purchased from Prolabo (WWR International, France). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Sigma (St. Louis, MO, USA). Phenolic standards were from Extrasynthèse (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Preparation of the extracts

Hydromethanolic extraction was performed stirring the sample (1 g) with 30 mL of methanol:water (80:20, *v/v*) at 25 ºC and 150 rpm for 1 h and filtered through Whatman No. 4

paper. The residue was then extracted with one additional 30 mL portion of the hydroalcoholic mixture. The combined extracts were evaporated at 35 ºC under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and then further lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA).

For infusion preparation the sample (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure. For decoction preparation the sample (1 g) was added to 200 mL of distilled water, heated (heating plate, VELP scientific) and boiled for 5 min. The mixture was left to stand for 5 min and then filtered under reduced pressure. The obtained infusions and decoctions were frozen and lyophilized.

Phenolic profile

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA), as previously described by the authors (Santos et al., 2013). Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were identified by comparing their retention time, UV-vis and mass spectra with those obtained from standard compounds, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal: catechin (y=158.42x+11.38, R^2=0.999); ellagic acid (y=32.748x+77.8, R^2=0.999); epicatechin (y=129.11x+11.663, R^2=0.9999); quercetin-3-*O*-glucoside (y=253.52x-11.615, R^2=0.999); isorahmetin-3-*O*-rutinoside (y=327.42x+313.78, R^2=0.999) and taxifolin (y=478.06x+657.33, R^2=0.999). For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compound from the same phenolic group. The results were expressed in mg per g of hydromethanolic extract or lyophilized infusion and decoction.

Antioxidant activity evaluation

The lyophilized hydromethanolic extracts, infusions and decoctions were re-dissolved in methanol:water (80:20, *v/v*) and water, respectively, to obtain stock solutions of 2.5 mg/mL. These solutions were further diluted to different concentrations to be submitted to the following assays. DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is

the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Santos et al., 2013). The final results were expressed in EC_{50} values (μg/mL), sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control.

Statistical analysis

For each plant material, three samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with α = 0.05. This treatment was carried out using SPSS v. 20.0 program.

3.2.2.3. Results and Discussion

Phenolic profile

Exemplificative phenolic profile of the hydromethanolic extract prepared from commercial and wild samples of *F. vesca* are shown in **Figure 12** and **Figure 13**. Peak characteristics (retention time, λ_{max} in the visible region, mass spectral data) and tentative identifications are presented in **Table 18**, whereas the quantification of both samples (hydromethanolic extracts, infusions and decoctions) is given in **Table 19**. Thirty-four phenolic compounds were identified, seventeen flavan-3-ols (*i.e.*, catechins and proanthocyanidins), ten ellagic acid/HHDP derivatives, three flavonols (*i.e.*, isorhamnetin and quercetin derivatives) and four dihydroflavonols (*i.e.*, dihydroquercetin derivatives).

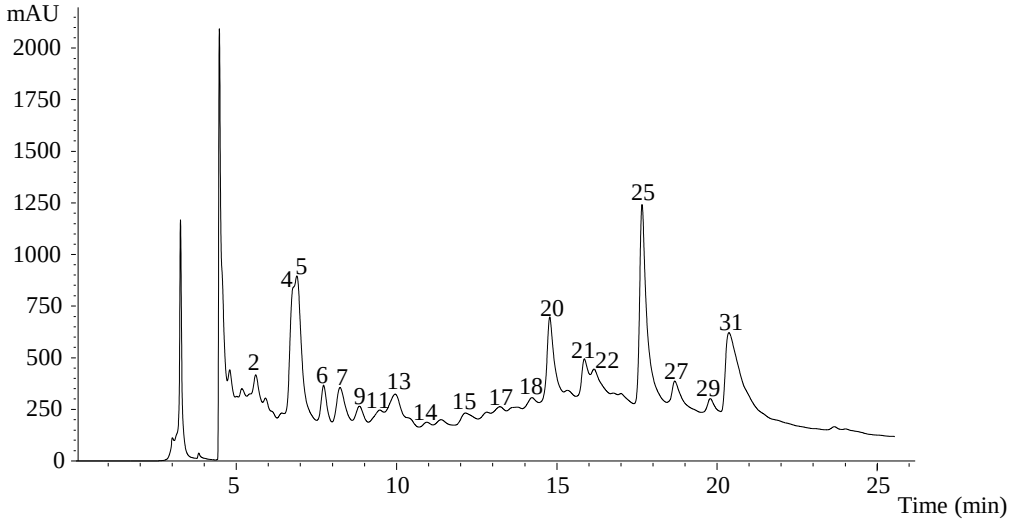

Figure 12. HPLC phenolic profile (obtained at 280 nm) of the hydromethanolic extract prepared from commercial *F. vesca* roots.

Flavan-3-ols

Peaks 1, 3-17 and 19 were tentatively identified as flavan-3-ol derivatives according to their UV spectra and pseudomolecular ions. Peaks 6 and 11 were positively identified as (+)-catechin and (-)-epicatechin, respectively, according to their retention time, mass and UV-vis characteristics by comparison with commercial standards. Peak 6 was the major phenolic compound found in the wild sample of *F. vesca*. Peak 1 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 451, releasing an MS^2 fragment at *m/z* 289 ($[M-H-162]^-$, loss of a hexosyl moiety), corresponding to a catechin monomer. This compound was tentatively identified as (epi)catechin hexoside, identity that was coherent with its earlier elution (higher polarity) compared with the parent aglycones (Peaks 6 and 11).

Proanthocyanidins (PAC) were assigned based on their pseudomolecular ions and MS^2 fragmentation patterns, characterised by the formation of product ions from the cleavage of the interflavan bond and retro-Diels-Alder (RDA) and heterocyclic ring fissions (HRF) of the elementary flavan-3-ol units (Friedrich et al., 2000; Gu et al., 2003). As for the cleavage of the interflavan bond, it has been reported that the terminal (lower) units of the PAC oligomer are released intact, while the extension (upper) units suffer a structural rearrangement yielding ions 2 Da lower than the original flavanol constituents (Friedrich et al., 2000; Gu et al., 2003). The analysis of the produced fragments provides information about the type elementary units and might also inform about their relative position in the PAC oligomer. Mass spectra do not allow, however, establishing the position of the linkage between flavanol units (*i.e.*, C4-C8 or C4-C6) nor differentiating between isomeric catechins (*e.g.*, catechin/epicatechin or afzelechin/epiafzelechin).

Peaks 3, 4 and 16 presented the same pseudomolecular ion $[M-H]^-$ at *m/z* 577 and MS^2 fragmentation patterns coherent with B-type (epi)catechin dimers (*i.e.*, (epi)catechin units with C4–C8 or C4–C6 interflavan linkages). Characteristic product ions were observed at *m/z* 451 (-126 mu), 425 (-152 mu) and 407 (-152-18 mu), attributable to the HRF, RDA and further loss of water from an (epi)catechin unit, and at *m/z* 289 and 287, that could be associated to the fragments corresponding to the lower and upper (epi)catechin unit, respectively. In the case of these three compounds comparison of their retention times with standards available in the laboratory allowed their tentative identification as the procyanidin dimers B3 (catechin-4,8-catechin), B1 (epicatechin-4,8-catechin) and B2 (epicatechin-4,8-epicatechin), respectively (Du et al., 2013; Pekic et al., 1998). Similarly, peaks 5, 9, 13 and 19 (pseudomolecular ions $[M-H]^-$ at *m/z* 865) and peaks 7 and 14 (pseudomolecular ions $[M-H]^-$ at *m/z* 1153) can be assigned as B-type (epi)catechin trimers and tetramers, respectively. In all cases, fragmentation patterns are coherent with those expected for such types of compounds, *i.e.*, similar at those observed for PAC dimers but with additional fragments from the alternative cleavages of different interflavan bonds. The same type of compounds have also been found and described in wild roots of *F. chiloensis* (Simirgiotis and Schmeda-Hirschmann, 2010) and fruits of *F. vesca* (Bubba et al., 2012; Sun et al., 2014).

Peak 10 showed an $[M-H]^-$ at *m/z* 561, consistent with the presence of an (epi)afzelechin and an (epi)catechin units. MS^2 fragments at *m/z* 435 and 407 can be ascribed to HRF and RDA cleavages of the (epi)catechin unit, whereas the observation of the ion at *m/z* 289 would suggest that this latter would be located in terminal position, so that the compound could be assigned as the dimeric properlagonidin (epi)afzelechin-(epi)catechin. The presence of a similar dimer in *F. vesca* berries was reported by Bubba et al. (2012).

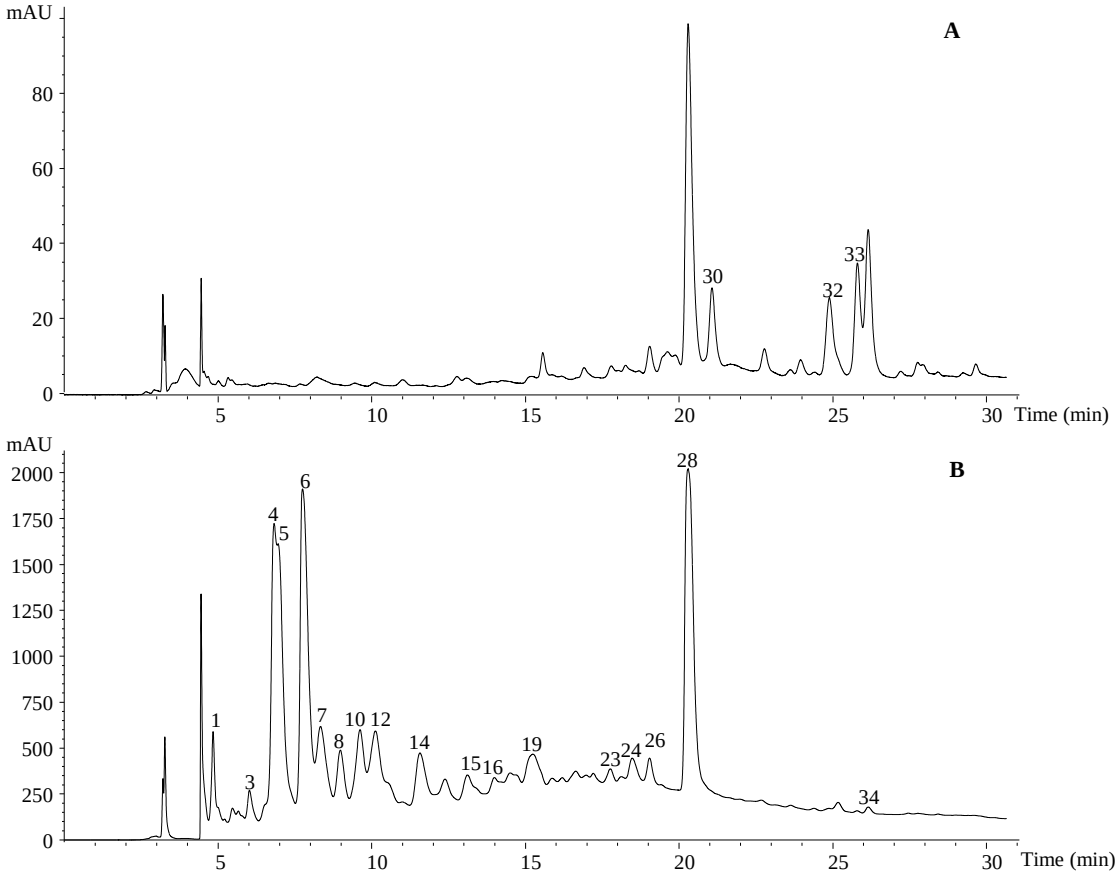

Figure 13. HPLC phenolic profile obtained at 370 nm (A) and 280 nm (B) of the hydromethanolic extract prepared from wild *F. vesca* roots..

Table 18. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, and tentative identification of phenolic compounds in *F. vesca* roots.

Peak	Rt (min)	λmax (nm)	Molecular ion [M-H]$^-$ (*m/z*)	MS^2 (*m/z*) (% of base peak)	Tentative identification
1	4.9	278	451	289(100)	(Epi)catechin hexoside
2	5.8	280	633	481(2), 463(2), 301(31)	Galloyl-HHDP-glucose
3	6.0	280	577	451(21), 425(47), 407(82), 289(82), 287(18)	Procyanidin dimers B3
4	7.0	278	577	451(31), 425(68), 407(100), 289(68), 287(15)	Procyanidin dimers B1
5	7.2	280	865	739(12), 713(16), 695(21), 577(26), 575(20), 425(10),407(17), 289(9), 287(14)	B-type (epi)catechin trimer
6	8.1	278	289	245(82), 203(49), 137(24)	(+)-Catechin
7	8.7	280	1153	865(6), 863(13), 577(11), 575(13), 289(8),245(3)	B-type (epi)catechin tetramer
8	8.9	278	849	577(23),559(57),289(26)	B-type (epi)afzelech-(epi)catechin-(epi)catechin
9	9.4	280	865	713(19),695(22),577(21),575(23),289(13),287(26)	B-type (epi)catechin trimer
10	9.6	278	561	435(48),407(42),289(86)	B-type (epi)afzelechin-(epi)catechin
11	10	280	289	245(91), 203(65)	(-)-Epicatechin
12	10.1	280	849	577(51),559(48),289(33),287(19)	B-type (epi)afzelech-(epi)catechin-(epi)catechin
13	10.6	280	865	713(16),695(32),577(18),575(19),289(11),287(25)	B-type (epi)catechin trimer
14	12.2	282	1153	865(11), 863(3), 577(8), 575(15), 289(8),245(2)	B-type (epi)catechin tetramer
15	13.5	278	833	561(25),543(77),407(8),289(70)	B-type (epi)afzelechin-(epi)afzelechin-(epi)catechin
16	13.9	280	577	451(28), 425(54), 407(7), 289(81), 287(11)	Procyanidin dimers B2
17	14.4	280	849	577(18),559(22),289(31),287(18)	B-type (epi)afzelech-(epi)catechin-(epi)catechin
18	15.1	278	935	633(15),301(16)	Galloyl-bis-HHDP-glucose isomer
19	15.2	280	865	713(9),695(21),577(18),575(),289(9),287(5)	B-type (epi)catechin trimer
20	15.7	272	1567	935(81),783(40),633(100), 613(2), 301(56)	Sanguiin h10 isomer
21	16.8	270	935	633(20),301(7)	Galloyl-bis-HHDP-glucose isomer
22	17.1	252/sh368	933	915(4),631(22),451(4)301(8)	Castalagin/Vescalagin
23	17.7	292	435	303(50),285(96),177(20),125(30)	Taxifolin-*O*-pentoside
24	18.5	292	435	303(24),285(63),177(24),125(29)	Taxifolin-*O*-pentoside
25	18.7	262	1567	935(100),783(73),633(53), 613(3), 301(21)	Sanguiin h10 isomer
26	19.0	292	435	303(32),285(85),177(12),125(32)	Taxifolin-*O*-pentoside
27	19.8	250/sh370	447	301(100)	Ellagic acid deoxyhexoside
28	20.3	292	435	303(69),285(84),177(35),125(30)	Taxifolin-3-*O*-arabinofuranoside
29	21.0	250/sh362	447	301(100)	Ellagic acid deoxyhexoside
30	21.1	356	463	301(100)	Quercetin-3-*O*-glucoside
31	21.8	254/366	301	284(14),256(8),229(10), 185(5)	Ellagic acid
32	24.9	356	433	301(100)	Quercetin-*O*-pentoside
33	25.8	350	477	315(100)	Isorhametin-*O*-hexoside
34	26.2	248/sh374	461	315(100),300(10)	Methy ellagic acid rhamnoside

Peaks 8, 12 and 17 showed an $[M-H]^-$ at *m/z* 849, consistent with the presence of one (epi)afzelechin and two (epi)catechin units. In all cases, no fragment at *m/z* 273 corresponding to the (epi)afzelechin unit was observed indicating that it was not located in terminal position. Similarly, the presence of that unit in middle position of the trimers must be also discarded owing to the production of the fragment at *m/z* 577 (-272 mu) from the loss of an (epi)afzelechin unit, indicating its position on the end of the structure. The fragment at *m/z* 559 would correspond to the (epi)afzelechin-(epi)catechin dimer produced after the loss of the terminal (epi)catechin unit, whereas this latter was observed as the ion at *m/z* 289. These compounds could be thus identified as B-type trimers consisting of (epi)afzelechin-(epi)catechin-(epi)catechin; the existence of different compounds can be explained by the presence of different catechin/afzelechin isomers and/or distinct interflavan linkages (C4-C8 or C4-C6). Similar propelargonidin trimers were also reported in fruits of *F. vesca* by Bubba et al. (2012) and Sun et al. (2014).

Peak 15 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 833, coherent with two (epi)afzelechin and one (epi)catechin units. Product ions were observed at *m/z* 561 (-272 mu, loss of an (epi)afzelechin unit), 543 (-272-18 mu, further loss of water), 407 (-272-154 mu, loss of an (epi)afzelechin unit + RDA cleavage of the (epi)catechin unit) and 289 (-272-272 mu, loss of two (epi)afzelechin units; terminal (epi)catechin unit), which identifying the peak as a B-type (epi)afzelechin-(epi)afzelechin-(epi)catechin trimer, also reported by Bubba et al. (2012).

Overall, the wild sample (mainly the infusion) showed higher contents of total flavan-3-ols in comparison with the commercial sample, mainly due to the presence of the compound (+)-catechin (peak 6; 65.07 mg/g). However in the commercial sample it was the decoction that presented the highest concentration of this type of compounds, due to the presence of the B-type (epi)catechin trimer (peak 5; 7.56 mg/g). Simirgiotis and Schmeda-Hirschmann (2010) described a similar flavan-3-ol profile in wild roots of *F. chiloensis*, mainly consisting of trimers and tetramers of (epi)catechin; however, the quantification of the individual compounds was not presented, so it cannot be compared.

Ellagic acid derivatives

These compounds were only quantifiable in the commercial sample of *F. vesca*; the wild sample only presented traces of these derivatives. Therefore, it can be concluded that the profile in ellagic acid derivatives is not specific of a plant species, depending on the cultivar and environmental factors with influence on the secondary metabolism.

Peak 2 presented an $[M-H]^-$ ion at *m/z* 633, presenting MS^2 fragment ions at *m/z* 481 (loss of a galloyl moiety, 152 mu), *m/z* 463 (loss of gallic acid, 170 mu) and *m/z* 301 ([M-H-

302]⁻), which is an evidence of the presence of an HHDP group in the molecule. A compound with similar characteristics was reported in *F. vesca* berries by Bubba et al. (2012) and in strawberry fruits by Gasperotti et al. (2013) that identified it as strictinin (i.e., galloyl-HHDP-glucose).

Peaks 18 and 21 were identified as bis-galloyl-HHDP-glucose isomers, presenting a pseudomolecular ion at *m/z* 935, with the main fragmentation ions at *m/z* 633 and *m/z* 301, corresponding to the loss of one HHDP unit and a galloyl-hexose unit, respectively. Similar compounds were reported in *F. vesca* fruits (Bubba et al., 2012; Sun et al., 2014) and identified as casuarictin/potentillin isomers. Peak 22 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 933 and fragment ions at *m/z* 915, 631, 451 and 301, in agreement with those attributed to castalagin or vescalagin isomers, previously reported in *F. vesca* (Bubba et al., 2012; Gasperotti et al., 2014). Peaks 20 and 25 were identified as sanguiin H-10 isomers, also reported in *F. vesca* by Bubba et al. (2012), presenting $[M-H]^-$ at *m/z* 1567 which produced a sequence of fragments, *m/z* 935 (loss of galloyl diHHDP glucose structure) followed by the characteristic fragments *m/z* 633 and 301. Peak 25 was the major ellagic acid derivative found in the hydromethanolic and aqueous extracts of the commercial sample.

Even though the above compounds (2, 18, 21, 22, 20 and 25) were previously reported in fruits of *F. vesca* (Bubba et al., 2012; Sun et al., 2013; Gasperotti et al., 2014), as well as in leaves and fruits of *F. chiloensis* (Simirgiotis and Schmeda-Hirschmann, 2010), this is the first time that they are described in roots of *F. vesca*.

Peaks 27, 29, 31 and 34 were assigned as ellagic acid derivatives, due to their UV-vis and mass spectra characteristics. Peak 31 was positively identified as ellagic acid, according to its retention, mass and UV-vis characteristics by comparison with commercial standard. Peak 27 and 29 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 447. Various compounds with similar UV and mass spectral characteristics were found in fruits of *F. vesca* (Bubba et al., 2012; Sun et al., 2014), strawberry (Gasperotti et al., 2013) and fruits and leaves of *F. chiloensis* (Simirgiotis and Schmeda-Hirschmann, 2010), and identified either as methylellagic acid pentosides or ellagic acid rhamnoside. In our case, the production of only one MS^2 fragment ion at *m/z* 301 (-146 mu, loss deoxyhexosyl moiety), corresponding to ellagic acid, suggested that they might be ellagic acid deoxyhexosides rather than methylellagic acid pentosides. Peak 34 possessed a molecular weight 15 mu higher than peaks 27 and 29, suggesting the presence of an additional methyl group. A similar compound was positively identified in *F. vesca* fruits based on mass, NMR and CD analyses by Gasperotti et al. (2013) as 3-*O*-methyl ellagic acid 3'-*O*-rhamnoside. To our knowledge this is the first time that these ellagic acid derivatives are described in *F. vesca* roots.

The distinct extracts of the commercial sample showed significant differences regarding ellagic acid derivatives, with hydromethanolic extracts presenting the highest concentration followed by decoction and infusion (16.06 mg/g, 3.88 mg/g and 2.81 mg/g, respectively).

Flavonols and dihydroflavonols

Peaks 23, 24, 26 and 28, all of them presenting a pseudomolecular ion $[M-H]^-$ at *m/z* 435, were identified as dihydroquercetin pentosides, based upon their UV spectra with λ_{max} at 292 nm and the production of an MS^2 fragment ion at *m/z* 303 (loss of a pentosyl moiety). Peak 28, the second major compound found in the wild sample, was tentatively assigned as taxifolin-3-*O*-arabinofuranoside, as that compound was previously reported as a major component in roots of *Fragaria x ananassa* (Ishimaru et al., 1995) and in fruits of *F. vesca* (Sun et al., 2014).

Peaks 30 and 32 presented UV spectra with λmax around 350 nm and an MS^2 product ion at *m/z* 301 indicating that they corresponded to quercetin derivatives. According to their pseudo molecular ions, they were identified as quercetin-3-*O*-glucoside (peak 30; $[M-H]^-$ at *m/*z 463), which was confirmed by comparison with a commercial standard, and quercetin-*O*-pentoside (peak 32; $[M-H]^-$ at *m/*z 433). Finally, peak 33 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 477 yielding a unique MS^2 fragment ion at *m/z* 315 (-162 mu; isorhamnetin), which was coherent with an isorhamnetin *O*-hexoside. The presence of quercetin-3-*O*-glucoside has been previously reported in *F. vesca* fruits (Sun et al., 2014), whereas a quercetin pentoside was described in roots of wild *F. chilloensis* (Simirgiotis and Schmeda-Hirschmann, 2010), however nothing was reported about *F. vesca* roots.

Contrary to proanthocyanidins and ellagic acid derivatives, flavonols and dihydroflavonols were only found in the wild sample. In fact, dihydroflavonols represented the second largest family of phenolic compounds found in the wild sample herein analysed, being at higher concentration in the decoction (32.39 mg/g) than in the infusion and the hydromethanolic extract (26.22 mg/g and 13.14 mg/g, respectively). Flavanols were also present in higher concentration in the decoction (0.58 mg/g) of the wild sample, followed by hydromethanolic and infusion extracts (0.53 mg/g and 0.50 mg/g, respectively). The fact that decoction extracts were the ones with the highest concentration of flavonols and dihydroflavonols could be due to the fact that high temperatures improve the efficiency of the extraction by increasing the solubility and diffusion coefficients of the compounds through the cell (Santos-Buelga et al., 2012). These types of compounds were also reported in roots of wild *F. chiloensis* (Simirgiotis and Schmeda-Hirschmann, 2010). The content of total flavonoids determined by those authors (0.55 g quercetin equivalents/100 g dw) was similar

to the one presented in the commercial sample of *F. vesca* roots studied herein, although lower than that found in the wild sample.

Table 19. Phenolic compounds quantification (mg/g) in the hydromethanolic extracts, infusions and decoctions obtained from commercial and wild samples of *F. vesca* (mean ± SD).

	Commercial samples			Wild samples		
Peak	**Hydromethanolic**	**Infusion**	**Decoction**	**Hydromethanolic**	**Infusion**	**Decoction**
1	-	-	-	4.11 ± 0.04	5.96 ± 0.17	6.34 ± 0.37
2	0.21 ± 0.001	tr	tr	-	-	-
3	-	-	-	2.26 ± 0.07	4.76 ± 0.05	4.76 ± 0.28
4	5.59 ± 0.119	5.828 ± 0.5	6.81 ± 0.293	20.3 ± 0.012	33.99 ± 0.24	34.98 ± 1.67
5	8.57 ± 0.261	8.76 ± 0.414	7.56 ± 0.414	20.77 ± 0.01	38.22 ± 0.19	31.31 ± 0.03
6	2.1 ± 0.086	2.38 ± 0.05	3.31 ± 0.201	39.26 ± 1.22	65.07 ± 1.19	56.95 ± 0.04
7	2.35 ± 0.088	2.49 ± 0.01	3.26 ± 0.199	10.38 ± 0.07	16.94 ± 0.55	9.85 ± 0.12
8	-	-	-	5.64 ± 0.20	9.14 ± 0.08	7.08 ± 0.35
9	1.64 ± 0.193	1.28 ± 0.048	1.29 ± 0.171	-	-	-
10	-	-	-	9.01 ± 0.01	14.87 ± 0.00	13.47 ± 0.05
11	1.51 ± 0.144	1.15 ± 0.072	1.9 ± 0.071	-	-	-
12	-	-	-	10.39 ± 0.02	16.58 ± 1.095	6.69 ± 0.73
13	3.43 ± 0.159	2.23 ± 0.03	3.53 ± 0.131	-	-	-
14	1.19 ± 0.032	0.7 ± 0.064	1.52 ± 0.126	7.11 ± 0.00	9.601 ± 0.66	7.82 ± 0.4
15	1.74 ± 0.139	1.57 ± 0.183	2.32 ± 0.226	3.27 ± 0.41	4.84 ± 0.37	4.19 ± 0.42
16	-	-	-	1.25 ± 0.01	2.19 ± 0.04	1.55 ± 0.00
17	2.03 ± 0.084	0.43 ± 0.036	0.61± 0.077	-	-	-
18	0.67 ± 0.032	tr	tr	-	-	-
19	-	-	-	3.38 ± 0.05	4.54 ± 0.03	4.16 ± 0.51
20	2.57 ± 0.13	0.06 ± 0.243	tr	-	-	-
21	0.87 ± 0.023	tr	tr	-	-	-
22	0.34 ± 0.02	tr	tr	-	-	-
23	-	-	-	0.26 ± 0.07	5.19 ± 0.03	6.19 ± 0.29
24	-	-	-	0.77 ± 0.05	1.27 ± 0.06	4.05 ± 0.09
25	6.58 ± 0.274	2.81 ± 0.008	2.72 ± 0.035	-	-	-
26	-	-	-	0.49 ± 0.044	0.15 ± 0.00	0.79 ± 0.10
27	0.52 ± 0.039	tr	tr	-	-	-
28	-	-	-	11.62 ± 0.06	19.59 ± 0.11	21.37 ± 0.35
29	tr	tr	tr	-	-	-
30	-	-	-	0.22 ± 0.00	0.27 ± 0.02	0.3 ± 0.01
31	4.29 ± 0.567	tr	1.17 ± 0.136	-	-	-
32	-	-	-	0.26 ± 0.00	0.23 ± 0.00	0.28 ± 0.01
33	-	-	-	0.05 ± 0.00	tr	tr
34	-	-	-	tr	tr	tr
TF3O	30.15 ± 0.46^{b}	26.82 ± 0.4^{c}	32.09 ± 1.36^{a}	137.13 ± 2.02^{c}	226.7 ± 2.12^{a}	189.17 ± 1.64^{b}
TED	16.06 ± 0.31^{a}	2.81 ± 0.01^{c}	3.88 ± 0.1^{b}	tr	tr	tr
TF	nd	nd	nd	0.53 ± 0.01^{b}	0.50 ± 0.02^{c}	0.58 ± 0.03^{a}
TdhF	nd	nd	nd	13.14 ± 0.22^{c}	26.22 ± 0.20^{b}	32.39 ± 0.07^{a}
TPC	46.21 ± 0.15^{a}	29.62 ± 0.38^{c}	35.97 ± 1.26^{b}	150.81 ± 1.8^{c}	253.42 ± 2.34^{a}	222.13 ± 1.58^{b}

TF3O- Total flavan-3-ols; TED- Total ellagic acid derivatives; TF- Total flavonols; TdhF- Total dihydroflavonols; TPC- Total phenolic compounds; tr- traces; nd- not detected. In each row, different letters mean significant statistical differences between samples ($p<0.05$).

Antioxidant activity

Data regarding antioxidant activity of the hydromethanolic extracts, infusions and decoctions obtained from commercial and wild samples of *F. vesca* roots are presented in **Table 20**. In general, wild samples gave lower EC_{50} values (higher antioxidant activity) than commercial samples. The exceptions were for β-carotene bleaching inhibition and TBARS assay, in which the hydromethanolic extracts and infusion (β-carotene bleaching) of commercial sample displayed the lowest EC_{50} value. In the commercial sample, the aqueous extracts gave the highest DPPH scavenging activity and reducing power (decoctions); and β-carotene bleaching inhibition (infusions). For TBARS assay, it was the hydromethanolic extract that presented the highest antioxidant activity (EC_{50}=6.69 μg/mL). In the wild sample, the aqueous extracts showed higher β-carotene bleaching and TBARS inhibition, while the hydromethanolic extract gave the highest reducing power (EC_{50}=40.98 μg/mL). For DPPH scavenging activity there were no significant differences between the hydromethanolic and aqueous extracts obtained from the wild sample.

The results obtained are similar to the ones described for the methanolic extracts of wild *F. chiloensis* ssp. *chiloensis* f. *chiloensis* roots (EC_{50} DPPH scavenging activity = 64.8 μg/mL; Simirgiotis and Schmeda-Hirschmann, 2010). However, Žugic et al. (2014) reported a lower EC_{50} value for DPPH scavenging activity of methanolic extracts of wild *F. vesca* leaves (13.46 μg/mL).

Correlations of total flavan-3-ols (TF3O), total flavonols and total dihydroflavonols (TF and TdhF, respectively; wild sample), total ellagic acid derivatives (TED; commercial sample) and total phenolic compounds (TPC), with the EC_{50} values obtained in the four antioxidant activity assays were performed. The wild sample showed high and positive correlation between TF3O, TdhF and TPC and β-carotene bleaching inhibition (R^2=0.7955, 0.7432 and 0.8537, respectively) and TBARS inhibition (R^2=0.8466, 0.876 and 0.9253, respectively). It also showed a high correlation between TdhF and reducing power assay (R^2=0.908). For the commercial sample, TF3O showed a high correlation with DPPH scavenging activity, β-carotene bleaching inhibition and TBARS inhibition (R^2=0.5451, 0.6856 and 0.7358, respectively). The fact that in the commercial sample TF3O correlated with DPPH assay might be related to the presence of B-type procyanidin (peaks 9 and 13) and propelargonidin trimers (peak 17), that were not present in the wild sample. Also in the commercial sample, TED showed a high and positive correlation with reducing power (R^2=0.9754), while TPC correlated with DPPH scavenging activity, reducing power and TBARS inhibition (R^2= 0.8676, 0.8176 and 0.5924, respectively). Low correlations for TED could be explained with the low concentration of these compounds in the commercial sample, when compared to the TPC contents.

Table 20. Antioxidant activity of hydromethanolic extracts, infusions and decoction of commercial and wild roots of *Fragaria vesca* (mean ± SD).

	Commercial samples			Wild samples			
EC_{50} values (μg/mL)	**Hydromethanolic**	**Infusion**	**Decoction**	**Hydromethanolic**	**Infusion**	**Decoction**	**Trolox**
DPPH scavenging activity	68.89 ± 2.29^{b}	255.81 ± 10.56^{a}	51.32 ± 0.88^{c}	50.03 ± 0.93^{a}	50.56 ± 1.07^{a}	50.62 ± 1.23^{a}	43.03 ± 1.71
Reducing power	327.75 ± 1.36^{a}	78.99 ± 2.87^{b}	67.92 ± 0.86^{b}	40.98 ± 1.17^{c}	44.78 ± 0.84^{b}	49.23 ± 0.18^{a}	29.62 ± 3.15
β-carotene bleaching inhibition	68.34 ± 6.73^{b}	23.44 ± 2.67^{c}	114.67 ± 7.00^{a}	116.26 ± 1.87^{a}	44.88 ± 4.55^{b}	66.10 ± 5.30^{b}	2.63 ± 0.14
TBARS inhibition	6.69 ± 0.79^{c}	24.25 ± 2.64^{a}	10.62 ± 0.75^{b}	35.76 ± 1.69^{a}	4.76 ± 0.30^{c}	6.14 ± 0.06^{b}	3.73 ± 1.9

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. In each row different letters mean significant statistical differences between samples ($p<0.05$).

Overall, the phenolic compounds profile of commercial and wild *F. vesca* roots presented some similarity regarding flavan-3-ols, being (epi)catechin derivatives (mainly, procyanidins), the major compounds found in both samples. Nonetheless, it could be observed that the commercial sample presented ellagic acid derivatives (mainly, ellagic acid glycosides), while the wild sample presented flavonols and dihydroflavonols (taxifolin derivatives).

The infusion of the wild sample gave the highest content of total phenolic compounds (253.42 mg/g) mainly due to flavan-3-ols (226.7 mg/g). Its decoction also gave the highest content of total dihydroflavonols (32.97 mg/g). It also showed higher DPPH scavenging activity, reducing power and TBARS inhibition. The high antioxidant capacity of the wild sample could be related to the presence of specific phenolic compounds, since high and positive correlations were obtained between TF3O, TF and TPC and β-carotene bleaching, and TBARS inhibition. The commercial sample showed higher content of total ellagic acid derivatives (mainly, the hydromethanolic extract; 46.21 mg/g) and higher β-carotene bleaching inhibition (mainly, the infusion). Although the roots of *F. vesca* are not widely known and used by the general public, this report shows its great antioxidant potential that could be displayed directly by consumption in infusions/decoctions or included in antioxidant formulations (hydromethanolic extract).

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Conflict of interest

The authors declare that they have no conflict of interest.

3.2.2.4. References

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3.2.3. Frutos silvestres de *Fragaria vesca* L.: uma fonte de fitoquímicos bioativos

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Wild *Fragaria vesca* L. fruits: a rich source of bioactive phytochemicals

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Abstract

Wild *Fragaria vesca* L. fruits were studied regarding nutritional and phytochemical compounds, as also antioxidant, antibacterial and biofilm formation inhibition activities. The fruits are good sources of carbohydrates (e.g., sucrose), soluble dietary fiber and polyunsaturated fatty acids, mainly linoleic and linolenic acids, as well as other components such as citric and succinic acids, vitamins B_9 and E (mainly γ-tocopherol). Significant

amounts of soluble sugars, citric acid and some amounts of ascorbic acid, vitamins B_9 and E (only α-tocopherol) were found also in the infusions. The hydromethanolic extracts revealed higher amounts of phenolic compounds, mainly ellagic acid derivatives and dihydroflavonol taxifolin-3-*O*-arabinofuranoside. Consistently, these extracts also showed higher antioxidant and antibacterial activities than the infusions, and were able to inhibit the formation of bacterial biofilm. Despite the lower content of bioactive compounds in the infusions compared to the fruits, both forms could be potentially applied in functional foods and/or nutraceuticals/pharmaceutical formulations.

Keywords: Wild strawberry fruits; Nutrients/phytochemicals; Antioxidant activity; Antibacterial activity; Biofilm inhibition.

3.2.3.1. Introduction

Fruits are raw material and used by people for food, either as edible products, or for culinary ingredients, for medicinal use or ornamental and aesthetic purposes. They are genetically very diverse group and play a major role in modern society end economy. Fruits are an important component of traditional food, but are also central to healthy diets of modern urban population[1–3]. The consumption of fruits is largely widespread throughout the world being the basis of most of the diets, not only for their nutritional characteristics, but also for the nutraceutical potential that they present [4]. Furthermore, there is an increasingly search for new sources of natural compounds with antioxidant and antimicrobial properties important for clinical applications [5,6] and food preservative purposes [7]. *Fragaria vesca* L., commonly known as wild strawberry or woodland strawberry, is an important fruit consumed worldwide. It belongs to the Rosaceae family and grows spontaneously in mountain zones, being also commonly found in roadsides and slopes [4,8]. As a wild plant, its productivity is lower than commercial varieties, however it is well known for its strongly flavored berries that are traditionally used in the preparation of sauces, jams, juices, syrups, dairy products and even liqueurs and cosmetic products [9–11]. *Fragaria vesca* fruits can be consumed either in fresh or in infusion preparations that are used in folk medicine for the treatment of intestinal disorders, also presenting diuretic and antidiarrheal properties [12,13]. It has also been proven that its polysaccharidic extract shows anticoagulant activity [12].

The study of the nutritional properties of foodstuffs is extremely important, since the synergistic effects between compounds can add other type of properties in addition to the nutritional ones, and for that reason a balanced diet containing such elements can provide the maintenance of human health [14]. The sugar composition in cultivated [4] and wild [15] *F. vesca* fruits has been studied, as well as organic acids [4,9,16], mineral [9] and dietary fiber composition [17]. Nevertheless, no complete studies on the nutritional and phytochemical

characterization of wild *F. vesca* fruits have been found in the literature. In particular, to the authors' best of knowledge, the composition on vitamins B_9 and C has never been reported. On the other hand, the study of its bioactive properties such as antioxidant and antimicrobial activities could open new opportunities of application in food, pharmaceutical or cosmetic sectors.

The bioactive properties of strawberry plant have been linked to the presence of phenolic compounds, mainly hydroxycinnamic and ellagic acid derivatives (e.g., ellagitannins), flavonols, anthocyanins and proanthocyanidins [18–25]. The antioxidant activity of *F. vesca* fruits has been studied [4,26], as well as the content in total phenolics [23,25–29] and phenolic composition, including anthocyanins [23,25,27,29]. Nonetheless, studies on the antimicrobial capacity and biofilm production inhibition of *F. vesca* fruits could not be found.

In the present work, a complete nutritional and phytochemical characterization of *F. vesca* fruits has been carried out. Furthermore, hydromethanolic extracts and infusions were prepared and evaluated for their antioxidant, antibacterial and biofilm formation inhibition activities, which were correlated with the composition in phenolic compounds.

3.2.3.2. Materials and methods.

Standards and Reagents

Acetonitrile, n-hexane and ethyl acetate were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Formic acid was purchased from Prolabo (VWR International, France). Fatty acids methyl ester (FAME) reference standard mixture (standard 47885-U) was purchased from Sigma-Aldrich (St. Louis, MO, USA), as well as trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), L-ascorbic acid, tocopherols, sugar and organic acid standards, nitric acid, hydrochloric acid, 5-CH_3-H_4folate monoglutamate (ref. 16252; Schircks laboratories, Jona, Switzerland), pteroyl diglutamic acid (ref. 16235; Schircks laboratories, Jona, Switzerland), pancreatic chicken homogenate (Pel Freeze, Rogers, Arkansas), rat serum, $NaBH_4$, formaldehyde and octanol. Micro and macroelement standards (> 99% purity), as well as $LaCl_2$ and CsCl (> 99% purity) were purchased from Merck (Darmstadt, Germany). Phenolic standards were from Extrasynthèse (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other general laboratory reagents were purchased from Panreac Química S.L.U. (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Samples and preparation of hydromethanolic extracts and infusions

The samples of wild *Fragaria vesca* L. fruits (harvested fully maturated) were collected in Serra da Nogueira (41° 43′ 12″ N, 6° 51′ 0″ W), Bragança, North-eastern Portugal, in July 2013. The fruits were conditioned in cooling boxes and transported to the laboratory. Voucher specimens (nº 9687) are deposited in the School of Agriculture Herbarium (BRESA) at the Polytechnic Institute of Bragança, Portugal. The samples were lyophilized (FreeZone 4.5, Labconco, Kansas, MO, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain homogenate sample.

For hydromethanolic extract preparations, each sample (1 g) was extracted by stirring with 30 mL of methanol/water (80:20 v/v, at 25 ºC at 150 rpm) for 1 h, followed by filtration through a Whatman filter paper No. 4. The residue was then extracted with an additional 30 mL portion of the hydromethanolic mixture and both extracts were combined. Afterwards, the extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and further lyophilized.

To prepare the infusions, each sample (500 mg) was added to 100 mL of boiled distilled water (pH 6.6) at 100 ºC and left to stand at room temperature for 5 min. Then the samples were filtered under reduced pressure (0.22μm), frozen and lyophilized for further analysis.

For anthocyanin extract preparation, the powdered sample (1 g) was extracted with 30 mL of methanol containing 0.5% trifluoroacetic acid (TFA), and filtered through a Whatman No. 4 paper. The residue was then re-extracted with an additional 30 mL portion of 0.5% TFA in methanol. The combined extracts were evaporated at 35 ^{o}C to remove the methanol, and re-dissolved in water. For purification, the extract solution was deposited into a C-18 SepPak® Vac 3cc cartridge (Phenomenex), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 10 mL of water and anthocyanins were further eluted with 5 mL of methanol:water (80:20, v/v) containing 0.1% TFA. The extract was concentrated under vacuum, lyophilized, re-dissolved in 1 mL of 20% aqueous methanol and filtered through a 0.22-□m disposable LC filter disk for HPLC analysis.

Nutritional value of the fruits

Proximate composition

The sample was analyzed for crude protein content (AOAC, 991.02), crude fat (AOAC, 989.05), carbohydrates and ash (AOAC, 935.42) according to the AOAC procedures [30]. Dietary fiber composition (AOAC, 993.19 and 991.42) were analyzed according to the method describe by Latimer et al. [31]. Total energy was calculated according to the following

equation: Energy (kcal/100 g) = 4 × (g proteins + g carbohydrates) + 2 × (g total dietary fiber) 9 × (g fat) [32].

Fatty acids

Fatty acids were determined by GC-FID (DANI model GC 1000 instrument, Contone, Switzerland) as previously described by [33,34] and the results were expressed as relative percentage of each fatty acid.

Chemical characterization of the fruits and infusions

Soluble sugars

Free sugars were determined by HPLC coupled to a RI detector (Knauer, Smartline system 1000, Berlin, Germany) using the internal standard (IS, melezitose) method or external standard method for infusions, as previously described by [33,34]. Results were expressed in g per 100 g of fresh weight of the fruits or in mg per 100 mL of infusion.

Organic acids

Organic acids were determined following a procedure previously described by [35] and [34] and the analysis was performed by ultra-fast liquid chromatography coupled to photodiode array detection (UFLC-PDA; Shimadzu Coperation, Kyoto, Japan), using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. Results were expressed in g per 100 g of fresh weight of the fruits or in mg per 100 mL of infusion.

Minerals

Mineral elements (930.05 of AOAC) analysis was performed according to a methodology previously described [34,36,37]. All measurements were performed in atomic absorption spectroscopy (AAS) with air/acetylene flame in Analyst 200 Perkin Elmer equipment (Perkin Elmer, Waltham, MA, USA), comparing absorbance responses with > 99.9% purity analytical standard solutions for AAS made with $Fe(NO_3)_3$, $Cu(NO_3)_2$, Mn $(NO_3)_2$, Zn $(NO_3)_2$, NaCl, KCl, $CaCO_3$ and Mg band.

Folates (*Vitamin* B_9)

Folate content was determined according to the methodology previously described by [34,38] and separation was performed using an HPLC (Ecom, Prague, Czech Republic), joined to an automatic injector (AS-1555, Jasco, Easton, MD, USA), and to a fluorescence detector (FP-2020, Jasco, Easton, MD, USA). The results were expressed in µg per 100 g of fresh weight of the fruits or in µg per 100 mL of infusion.

Tocopherols (*Vitamin E*)

Tocopherols were determined following a procedure previously described by [33,34], using a HPLC system (Knauer, Smartline system 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, USA) programmed for excitation at 290 nm and emission at 330 nm, using the IS (tocol) method for quantification. The results were expressed in µg per 100 g of fresh weight of the fruits or in µg per 100 mL of infusion.

Individual phenolic profile and bioactive properties of fruits hydromethanolic extracts and infusions

Phenolic compounds analysis

Phenolic profile was determined in the lyophilized extracts and infusions re-dissolved in methanol:water (80:20, *v/v*) and pure water, respectively, by HPLC-DAD-MS/ESI (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA), as previously described [39–41]. Double online detection was carried out with a diode array detector (DAD, 280 and 370 nm as the preferred wavelengths) connected in line with a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany). The identification of the different phenolic compounds was performed by comparison with available commercial standards, or tentatively identified using reported data from literature. For quantitative analysis, a calibration curve for each available phenolic standard was created, when no commercial standard was available a similar compound from the same phenolic group was used as a standard. The results were expressed in mg per g of lyophilized extract or infusion.

Anthocyanins analysis

Anthocyanins were determined in the lyophilized extracts and infusions (re-dissolved in methanol:water (80:20, *v/v*) and pure water, respectively) by HPLC (Hewlett-Packard 1100) as previously described [39]. Double online detection was carried out in a DAD, using 520 nm as the preferred wavelength, and in an MS connected to the HPLC system via the DAD cell outlet. The identification of the different anthocyanins was performed by comparison with available commercial standards, or tentatively identified using reported data from literature. For quantitative analysis, a calibration curve for each available anthocyanin standard was constructed; when no commercial standard was available a similar compound was used as a standard. The results were expressed in µg per g of lyophilized extract or infusion.

Antioxidant activity evaluation

The lyophilized extracts and infusions were re-dissolved in methanol:water (80:20, *v/v*) and water, respectively, to obtain stock solutions of 2.5 mg/mL, which were further diluted to obtain a range of concentrations for antioxidant activity evaluation by DPPH radical-scavenging activity, reducing power, inhibition of β-carotene bleaching and lipid peroxidation inhibition in porcine brain homogenates (TBARS) [33,40,41]. The final results were expressed as EC_{50} values (μg/mL), sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control.

Antibacterial activity evaluation

The microorganisms used were clinical isolates from patients hospitalized in various departments of the Local Health Unit of Bragança and Hospital Center of Trás-os-Montes and Alto-Douro Vila Real, Northeast of Portugal (supplementary material).

MIC determinations were performed by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay following the methodology described by the authors [42]. MIC was defined as the lowest extract concentration that prevented this change and exhibited inhibition of bacterial growth.

The biofilm assay was carried out adapting the protocol described by the authors [42]. Results for this test were given as percentage of biofilm formation inhibition applying the following formula:

Biofilm formation inhibition percentage = 100 − (ODassay/ODcontrol) x 100

Statistical analysis

Three different samples were used and all the extractions and assays were performed in triplicate. The results were expressed as mean values and standard deviation (SD), being analysed using a Student´s t-test, with α = 0.05 (SPSS v. 22.0 program, IBM Corp., Armonk, NY, USA).

3.2.3.3. Results and Discussion

Nutritional composition of F. vesca fruits

Results regarding the proximate composition, dietary fiber and fatty acids content of wild *F. vesca* fruits are presented in **Table 21**. Carbohydrates and dietary fiber were the major macronutrients, followed by fat, ash and protein. In terms of dietary fiber content, soluble dietary fiber (mainly pectins) was the predominant one, with a content higher than the

one described by Ramulu and Rao [17] in *F. vesca* fruits from India (0.7 g/100 g fw). It is described that the daily consumption of fiber has a beneficial health effect, mainly in the digestive tract or even on the prevention of diabetes; especially soluble dietary fiber has a very large impact on the level of fat and arteriosclerosis in humans. The current recommended consumption of total dietary fiber is estimated to be 20 g/person/day, so that the consumption of just 100 g of fresh wild strawberry would cover almost a third of the recommended intake [43]. Regarding fatty acids profile, 13 different compounds were identified, being notorious a predominance of polyunsaturated fatty acids, mainly due to the presence of linolenic (C18:3n3), γ-linolenic (C18:3n6) and linolenic (C18:2n6) acids.

Table 21. Nutritional value, dietary fiber and fatty acids content in fruits of wild *Fragaria vesca* L. (mean ± SD).

Nutritional value (g/100 g fw)	
Moisture	81.72 ± 0.01
Fat	0.61 ± 0.01
Proteins	0.51 ± 0.01
Ash	1.00 ± 0.01
Total available carbohydrates	10.42 ± 0.23
Total dietary fiber	5.78 ± 0.21
Energy (kcal/100 g fw)	56.13 ± 0.69
Dietary fiber (g/100 g fw)	
Soluble dietary fiber	5.25 ± 0.17
Insoluble dietary fiber	0.62 ± 0.07
Fatty acids (relative percentage)	
C10:0	0.02 ± 0.002
C12:0	0.03 ± 0.002
C14:0	0.05 ± 0.004
C15:0	0.02 ± 0.003
C16:0	2.76 ± 0.06
C18:1n9	1.24 ± 0.013
C18:2n6	10.59 ± 0.07
C18:3n6	40.06 ± 0.24
C18:3n3	43.37 ± 0.14
C20:1	1.00 ± 0.06
C20:2	0.24 ± 0.01
C20:3n6	0.23 ± 0.03
C22:1n9	0.39 ± 0.03
SFA	2.88 ± 0.07
MUFA	2.63 ± 0.10
PUFA	94.49 ± 0.04

The results are expressed on fresh weight basis. C10:0- capric acid, C12:0- lauric acid, C14:0- myristic acid, C15:0- pentadecanoic acid, C16:0- palmitic acid, C18:1n9- oleic acid, C18:2n6- linoleic acid, C18:3n3- linolenic acid, C18:3n6- γ-linolenic acid methyl ester, C20:0- arachidic acid, C20:1- cis-11-eicosenoic acid, C20:2- cis-11,14-eicosadienoic acid, C20:3n6- cis-8,11,14-eicosatrienoic acid, C22:1n9- erucic acid; SFA- saturated fatty acids, MUFA- monounsaturated fatty acids, PUFA- polyunsaturated fatty acids.

Chemical composition of F. vesca fruits and infusions

The results of the composition of *F. vesca* fruits and infusions in soluble sugars, organic acids, mineral elements, folates and tocopherols are given in **Table 22**. The profile was very similar, expect for tocopherols. Sucrose was the major soluble sugar found in the fruits and in the infusions, followed by fructose and glucose. Very similar contents were reported by Doumett et al.[4] in various cultivars of *F. vesca* fruits from Italy and by Ornelas-Paz et al.[16] in *Fragaria x ananassa* Duch, Cv. Albion from Mexico; however, lower contents were described by Blanch et al.[15] in *Fragaria vesca* cv. Mara de Bois fruits from Spain (sucrose = 1.49 g/100 g fw).

Citric and succinic acids were the most abundant organic acids in the fruits and infusions [4]; citric acid was also described as the major organic acid in *F. vesca* fruits but in lower levels (1.29 g/100 g fw), followed by malic acid, while no more organic acids were detected. The same was observed in *F. vesca* fruits from Italy [9]. Ornelas-Paz et al.[16] described citric acid as the major one, followed by malic acid, and also with the presence of ascorbic acid in cultivars of *Fragaria x ananassa*.

Related to mineral composition, the microelements found in higher amounts in both samples were manganese (Mn), followed by iron (Fe) and zinc (Zn). Copper (Cu) was not detected in the studied samples, however, Caruso et al.[9] described the presence of copper in hydroponic cultures of *F. vesca* fruits. Regarding macroelements, potassium (K) was the major one in the fruits, while calcium (Ca) was the most prevalent macroelement in the infusions. Magnesium (Mg) was also present in both samples. The mineral elements concentration in infusions depends mainly on three factors: the linkages to the plant cell tissues, mainly in the insoluble dietary fiber fraction, the solvent employed for extraction and the temperature used to prepare the infusions that could help breaking down the connection between minerals and cell constituents, influence the extraction yield of these elements [44]. Folates (Vitamin B_9) were also detected both in fruits and in the corresponding infusions [34] also detected folates in the infusions of wild roots and vegetative parts of wild *F. vesca*, but in higher amounts. The folate content was also determined in other fruits, such as coconut and pineapple, but it was found in significant lower amounts (10.0 and 10.5 μg/100 g fw, respectively) [45]. The recommended daily intake for folates is 200 μg/day, according to the EC Regulation number [29], which leads to the conclusion that the daily consumption of 100 g of fresh fruit or 100 mL of its infusion would cover 15% and 2% of the recommended intake, respectively. In terms of tocopherols which are mainly found in the seeds, the four forms were quantified in the fruits, being γ-tocopherol the main one, followed by α-tocopherol. In the infusions, only α-tocopherol was found, but not γ-tocopherol, which may be due to the different stability of the compounds under heat treatment. Britz et al. [43] observed that α-

tocopherol had a tendency to increase at high temperatures after thermal treatment in brown rice, whereas the opposite was observed for γ-tocopherol.

Table 22. Soluble sugars, organic acids, minerals, folates and tocopherols content in wild *Fragaria vesca* L. fruits and infusions (mean ± SD).

	Fruits	Infusions
Soluble sugars	**g/100 g fw**	**mg/100 mL**
Fructose	1.60 ± 0.01	33.43 ± 0.80
Glucose	1.44 ± 0.01	30.07 ± 0.42
Sucrose	3.20 ± 0.02	66.44 ± 1.50
Raffinose	0.070 ± 0.001	1.32 ± 0.02
Sum	6.31 ± 0.03	131.26 ± 2.75
Organic acids	**g/100 g fw**	**mg/100 mL**
Oxalic acid	0.040 ± 0.001	tr
Malic acid	0.74 ± 0.01	1.024 ± 0.001
Ascorbic	0.040 ± 0.001	tr
Citric acid	5.59 ± 0.04	25.98 ± 0.002
Succinic acid	1.14 ± 0.04	5.72 ± 0.01
Sum	7.55 ± 0.01	32.7 ± 0.3
Microelements	**mg/100 g fw**	**mg/100 mL**
Fe	0.72 ± 0.01	0.059 ± 0.001
Mn	1.27 ± 0.09	0.106 ± 0.002
Zn	0.19 ± 0.01	0.034 ± 0.001
Macroelements	**mg/100 g fw**	**mg/100 mL**
Ca	11.8 ± 0.3	4.4 ± 0.2
Mg	2.9 ± 0.2	3.64 ± 0.23
K	18.7 ± 0.5	2 ± 0.1
Folate (Vitamin B_9)	**µg/100 g fw**	**µg/100 mL**
	29.33 ± 0.35	4.044 ± 0.001
Tocopherols	**mg/100 g fw**	**µg/100 mL**
α-Tocopherol	0.50 ± 0.01	0.30 ± 0.02
β-Tocopherol	0.050 ± 0.001	nd
γ-Tocopherol	1.52 ± 0.01	nd
δ-Tocopherol	0.29 ± 0.01	nd
Sum	2.35 ± 0.01	0.30 ± 0.02

The results in fruits are expressed on fresh weight basis; nd- not detected; tr- traces (< LOQ: 42 µg/mL quinic acid and 50 µg/mL for ascorbic acid); Fe- iron Cu- cooper, Mn- manganese, Zn- zinc, Ca- calcium, Mg- magnesium, K- potassium. Calibration curves for organic acids: oxalic acid (y=9x106x + 377946, $R2$=0.994); malic acid (y =863548x + 55571, $R2$=0.999); ascorbic acid (y =108x + 751815, $R2$=0.998); citric acid (y =106x + 16276, $R2$=1); succinic acid (y =603298x + 4994.1, $R2$=1).

Individual phenolic profile in F. vesca hydromethanolic extracts and infusions

Table 23 presents the peak characteristics (retention time, λ_{max} in the visible region, mass spectral data), tentative identifications and quantification of phenolic compounds in hydromethanolic extracts and infusions prepared from wild *F. vesca* fruits. An exemplificative phenolic profile of the hydromethanolic extracts is shown in **Figure 14A** and **B**. Thirty-two phenolic compounds were identified, one phenolic acid, twenty-two ellagic acid/HHDP derivatives, two flavan-3-ols, one dihydroflavonol and six anthocyanins.

Peak 10 was the only phenolic acid derivative found in *F. vesca* fruits, being tentatively identified as ferulic acid di-hexoside, presenting a pseudomolecular ion $[M-H]^-$ at

m/z 517 releasing an MS^2 fragment at *m/z* 193, attributed to a ferulic acid and corresponding to the loss of two hexose moieties [M-H-162-162]$^-$. Peaks 6 and 8 were the only detected flavan-3-ol, being tentatively identified as procyanidin dimer B1 and (+)-catechin, respectively, which were previously reported in *F. vesca* fruits [23] and in *F. vesca* roots and vegetative parts [40,41] Peak 22 was identified as the dihydroflavonol taxifolin-3-*O*-arabinoside based on its molecular ion and fragmentation pattern, as previously described in the roots of wild *F. vesca* [40].

As for *F. vesca* roots [40] and vegetative parts [41], ellagic acid derivatives represent the largest group of phenolic compounds identified in *F. vesca* fruits, although these latter revealed lower concentrations. This can be explained by the fact that such compounds have a preferred tendency to accumulate in certain types of tissues, such as leaves and roots, rather than in fruit tissues [18], as well as to the greater moisture content existing in the fruits. Ellagic acid rhamnosides (peaks 19 and 21), ellagic acid (peak 23) and dimethyl ellagic acid pentosides (peaks 25 and 26) were previously reported in roots and vegetative parts of *F. vesca* [40,41]. Peaks 13 ([M − H]$^-$ at *m/z* 463) and 18 ([M − H]$^-$ at *m/z* 433) showed UV spectra similar to ellagic acid and an MS^2 fragment at *m/z* 301 (ellagic acid) from the losses of 162 mu and 132 mu, respectively, being tentatively identified as ellagic acid hexoside and ellagic acid pentoside, respectively. Similarly, peaks 20 ([M − H]$^-$ at *m/z* 477) and 24 ([M − H]$^-$ at *m/z* 447) were tentatively identified as methyl ellagic acid hexoside and pentoside, respectively. Both peaks presented a MS^2 fragment at *m/z* 315, corresponding to the loss of an hexosyl ([M–H-477-301]$^-$; 162 mu) and pentosyl moiety ([M–H-447-301]$^-$; 132 mu), respectively, and also a second fragment ion at *m/z* 301 (ellagic acid), pointing to the further loss of a methyl group.

The remaining compounds correspond to hydrolysable tannins, namely bis-HHDP-glucose isomers (peaks 1 and 2), galloyl-HHDP-glucose (peak 7), galloyl-bis-HHDP-glucose isomers (peaks 12, 14 and 16), castalagin/vescalagin (peak 15) and Sanguiin h10 (peak 17). All these compounds were previously reported in *F. vesca* roots and vegetative parts [40,41], as well as by other authors in fruits of *F. vesca* [23–25] and *F. chiloensis* spp. [21]. Sanguiin h10 (peak 17) was the main compound found in the hydromethanolic extracts and infusions of the fruits, as also reported by [34,40]. Peaks 3 and 5 ([M-H]$^-$ at *m/z* 951) released MS^2 fragments at 907, 783 and 301, corresponding to the loss of a carboxylic group (44 mu), a gallic acid unit (168 mu) and the tris-galloyl-hexoside residue (488+162 mu), respectively, being therefore tentatively identified as two tris-galloyl-HHDP hexose isomers, already reported in fruits of *F. vesca* by [23]. Peak 11 ([M−H]$^-$ ion at *m/z* 785) presented MS^2 fragment ions at *m/z* 615 (loss of gallic acid, 170 mu), *m/z* 463 (further loss of a galloyl moiety, 152 mu) and *m/z* 301 (loss of an hexose residue, 162 mu), being tentatively identified as digalloyl-HHDP-hexose. This

compound was previously reported in fruits of *F. vesca* [23,24]. Finally, peaks 4 ($[M-H]^-$ at *m/z* 663) and 9 ($[M-H]^-$ at *m/z* 965) could not be identified, although they corresponded to ellagitannins, as revealed by their UV spectra and the MS^2 fragment ions observed at *m/z* 481 (HHDP-hexose unit) and 301 (ellagic acid). An unknown ellagitannin with the same characteristics as peak 9 was previously found by [47] in leaves of *F. vesca*. Peaks 27-32 corresponded to anthocyanins found in *F. vesca* fruits. Cyanidin-3-*O*-glucoside (peak 27), pelargonidin-3-*O*-glucoside (peak 28) and peonidin-3-*O*-glucoside (peak 29) were identified according to their retention, mass and UV-vis characteristics and comparison with commercial standards. Peaks 30-32 showed molecular weights 86 Da greater than the previous compounds, which allowed their tentative identification as the corresponding malonyl derivatives. All these anthocyanins have been already reported in *F. vesca* berries by [23]. Pelargonidin-3-*O*-glucoside was the major anthocyanin found in both extracts, whereas the malonyl derivatives were only detected in the hydromethanolic extracts, maybe due to their lower polarity comparing with the parent glucosides and/or a less efficient extraction in the case of infusions.

Table 23. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification, phenolic (mg/g) and anthocyanin (µg/g) compounds quantification in wild *Fragaria vesca* L. fruits.

Peak	Rt (min)	λmax (nm)	[M-H]$^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Hydromethanolic extracts	Infusions	*t*-Students test *p*-value
Phenolic compounds								
1	4.8	276	783	481(13),301(27)	Bis-HHDP-glucose isomer	0.5 ± 0.1	nd	-
2	5.1	248	783	481(9),301(17)	Bis-HHDP-glucose isomer	0.32 ± 0.02	nd	-
3	5.4	258	951	907(61),783(24),301(11)	Trigalloyl HHDP hexose	0.61 ± 0.02	nd	-
4	5.7	264	663	481(100),301(44)	Unknown ellagitannin	0.26 ± 0.04	nd	-
5	6.14	280	951	907(78),783(20),301(10)	Tris-galloyl-HHDP hexose	0.27 ± 0.02	nd	-
6	7.13	272	577	451(33),425(529),407(93),289(68),287(10)	Procyadinin dimer B1	1.56 ± 0.01	nd	-
7	7.2	280	633	481(2),463(14),301(100)	Galloyl-HHDP-glucose	0.9 ± 0.1	1.5 ± 0.2	<0.001
8	8.2	278	289	245(73),203(47),137(37)	(+)-catechin	2.8 ± 0.4	nd	-
9	11.1	284	965	783(22),481(16),301(9)	Unknown ellagitannin	0.6 ± 0.1	nd	-
10	11.7	326	517	193(100),134(9)	Ferulic acid di-hexoside	0.40 ± 0.04	nd	-
11	13.2	278	785	615(11),463(3),301(46)	Digalloyl-HHDP-hexose	0.68 ± 0.04	0.8 ± 0.2	0.001
12	15.4	312	935	633(17),301(23)	Galloyl-bis-HHDP-glucose isomer	1.0 ± 0.3	1.4 ± 0.3	0.002
13	15.5	254/sh358	463	301(100)	Ellagic acid hexoside	0.4 ± 0.1	nd	-
14	15.8	276	935	783(2),633(15),301(16)	Galloyl-bis-HHDP-glucose isomer	2.6 ± 0.3	1.9 ± 0.2	0.968
15	17.1	254/sh336	933	631(17),301(33)	Castalagin/Vescalagin	1.5 ± 0.1	nd	-
16	18.3	262	935	783(38),633(8),301(15)	Galloyl-bis-HHDP-glucose isomer	1.06 ± 0.04	nd	-
17	18.9	278	1567	935(100),783(4),633(6),613(4)	Sanguiin h10	13.7 ± 0.5	5.4 ± 0.3	<0.001
18	19.3	250/sh366	433	301(100)	Ellagic acid pentoside	3.0 ± 0.2	1.6 ±0.2	<0.001
19	19.6	252/sh360	447	301(100)	Ellagic acid rhamnoside	0.23 ± 0.01	nd	-
20	19.8	246/sh362	477	315(679,301(19)	Methyl ellagic acid hexoside	0.29 ± 0.03	nd	-
21	20.3	254/sh364	447	301(100)	Ellagic acid rhamnoside	0.61 ± 0.04	nd	-
22	21.07	292	435	303(49),285(84),177(21),125(30)	Taxifolin-3-*O*-arabinofuranoside	7.0 ± 0.4	2.3 ± 0.1	<0.001
23	21.12	254/sh368	301	284(7),185(4)	Ellagic acid	1.7 ± 0.2	1.9 ± 0.3	0.110
24	23.9	246/sh376	447	315(90),300(35)	Methyl ellagic acid pentoside	0.32 ± 0.04	nd	-
25	25.6	262/sh378	461	315(100),301(1)	Dymethyl ellagic acid pentoside	6.7 ± 0.1	2.7 ± 0.2	<0.001
26	27.4	250/sh366	461	315(100),301(18)	Dymethyl ellagic acid pentoside	0.6 ± 0.1	0.3 ± 0.1	<0.001
					Total phenolic acids	**0.40 ± 0.04**	**nd**	-
					Total ellagic acid derivatives	**37.9 ± 0.4**	**17.5 ± 0.4**	<0.001
					Total flavan 3-ols	**4.4 ± 0.3**	**nd**	-
					Total dihydroflavonols	**7.0 ± 0.4**	**2.3 ± 0.4**	<0.001
					Total phenolic compounds	**49.7 ± 0.4**	**19.8 ± 0.5**	<0.001

Anthocyanin compounds

Peak	Rt (min)	λ_{max} (nm)	$[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Hydromethanolic extracts	Infusions	*t*-Students test *p*-value
27	16.6	514	449	287(100)	Cyanidin-3-glucoside	2.6 ± 0.1	0.304 ± 0.002	<0.001
28	19.34	504	433	271(100)	Pelargonidin-3-glucoside	4.6 ± 0.2	0.477 ± 0.004	<0.001
29	21.83	518	463	301(100)	Peonidin-3-glucoside	0.48 ± 0.01	0.084 ± 0.001	<0.001
30	26.67	518	535	449(2),287(100)	Cyanidin-malonylglucoside	0.30 ± 0.02	nd	-
31	30.57	504	519	433(2),271(100)	Pelargonidin-malonylglucoside	0.60 ± 0.04	nd	-
32	32.55	518	549	301(100)	Peonidin-malonylglucoside	0.11 ± 0.01	nd	-
					Total Anthocyanins	**9.02 ± 0.03**	**0.86 ± 0.01**	<0.001

Standard calibration curves: catechin (y =158.42x+11.38, $R2$=0.999); cyanidin-3-O-glucoside (y=630276x+153.83, $R2$=0.999); ellagic acid (y=36.466x+35.44, $R2$=0.999); ferulic acid (y=525.36x+233.82, $R2$=0.999); pelargonidin-3-O-glucoside (y=268748x+71.423, R^2=0.999); peonidin-3-O-glucoside (y=537017x+71.469, R^2=0.999); taxifolin (y=224.31x+148.41, $R2$=0.999).

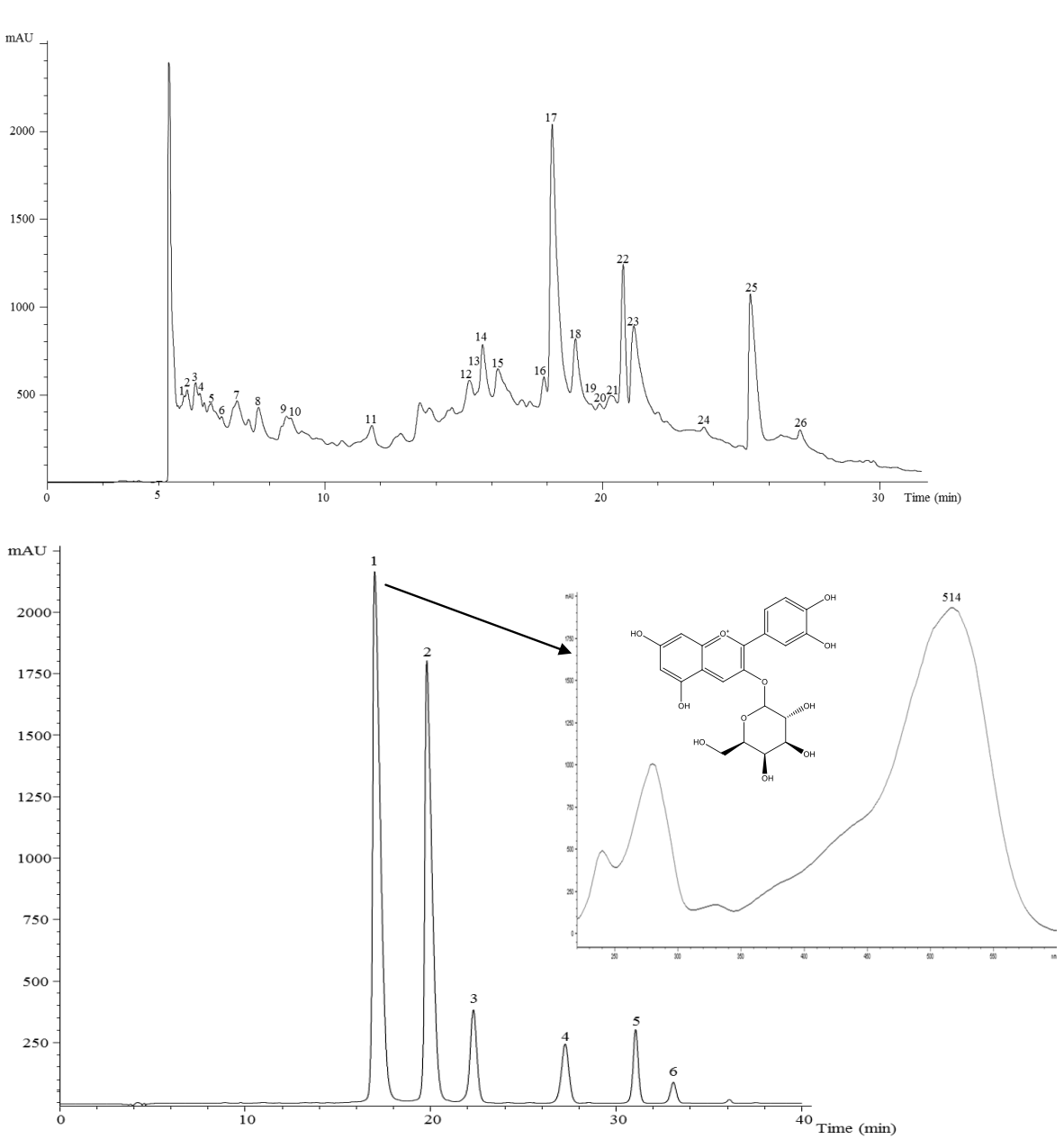

Figure 14. HPLC phenolic profile obtained at 280 nm (**A**) and 520 nm (**B**) of the hydromethanolic extract prepared from wild *Fragaria vesca* L. fruits

Antioxidant and antibacterial activity of F. vesca hydromethanolic extracts and infusions

Data regarding the antioxidant and antibacterial activity of the hydromethanolic extracts and infusions obtained from wild *F. vesca* fruits, are presented in **Table 24**. It is clearly evident the higher antioxidant capacity of the hydromethanolic extracts in comparison with the infusions, observed in all the performed assays.

By analysing **Table 24** it was verified that both hydromethanolic extracts and the infusions showed antibacterial activity against all Gram positive and Gram negative bacteria

tested, including those with high antibiotic susceptibility and with extended spectrum betalactamase (*Escherichia coli* ESBL 1 and 2 and *Klebsiella pneumoniae* ESBL). It should also be noted the significant MIC values observed for bacteria associated with health care such as MRSA, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The hydromethanolic extracts showed also higher antibacterial activity than the infusions, presenting lower MIC values for the Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*.

The biofilm assay was only performed for the hydromethanolic extracts, owing to their higher phenolics contents and antioxidant and antibacterial activities compared with the infusions. The extracts showed capacity to inhibit the formation of biofilm in *E. coli* ESBL 1*, E. coli* ESBL 2, *Klebsiella pneumoniae* ESBL and MRSA, presenting percentages of inhibition for each bacteria of 47%, 49%, 62% and 85%, respectively.

Correlation of total phenolic acids (TPA), total ellagic acid derivatives (TED), total flavan-3-ols (TF3O), total dihydroflavonols (TDF), total phenolic compounds (TPC) and total anthocyanins (TA) with the EC_{50} values obtained in the four antioxidant activity assays and the MIC values obtained in the antibacterial activity assay were performed (**Table 24**). The results showed high correlations with all the phenolic compound families found in both hydromethanolic extracts and infusions of *F. vesca* fruits. The best results were obtained for reducing power and TBARS inhibition with TPA (r^2=0.9929 and 0.9916, respectively), TED (r^2=0.9967 and 0.9954, respectively), TF3O (r^2=0.995 and 0.9937, respectively), TPC (r^2=0.9972 and 0.9958, respectively) and TA (r^2=0.998 and 0.9966, respectively). For the antibacterial activity assay the same families of phenolic compounds showed the best results for *E. coli* and *P. aeruginosa* with TPA (r^2=0.9938), TED (r^2=0.9976), TF3O (r^2=0.9959), TPC (r^2=0.9959) and TA (r^2=0.9989). These results are in accordance with other authors that proved the correlation between the presence of phenolic compounds and antimicrobial activity in natural extracts [48].

In conclusion, the fruits of wild *F. vesca* represent a good source of carbohydrates, soluble dietary fiber and polyunsaturated fatty acids, mainly linoleic and linolenic acids. They also showed to be a good source of sucrose, citric and succinic acid, vitamin B_9 and vitamin E (mainly γ-tocopherol). Their infusions presented significant amounts of soluble sugars (sucrose and glucose) and citric acid, as well as some levels of folates and vitamin E (only α-tocopherol) and trace amounts of ascorbic acid. Regarding phenolic composition, the hydromethanolic extracts showed much higher amounts than the infusions, being ellagic acid derivatives (especially sanguiin h10) and dihydroflavonols (taxifolin-3-*O*-arabinofuranoside) the majority individual compounds. The hydromethanolic extracts also revealed higher antioxidant and antibacterial activity than the infusions, and also proved to have the capacity

to inhibit the biofilm formation. These bioactivities were highly correlated with the presence of phenolic compounds. Despite the lower contents of bioactive compounds in infusions of wild *F. vesca* compared to its fruits, the results obtained are of great novelty since both forms could be potentially applied in novel food products such as functional foods (infusions) and/or nutraceuticals/pharmaceutical formulations (hydromethanolic extracts).

Table 24. Antioxidant and antimicrobial activity of the hydromethanolic extract and infusion obtained from wild *Fragaria vesca* L. fruits and their correlation factor (r^2) with the phenolic compounds families identified.

	Hydromethanolic extracts	Infusions	*t*-Students test *p*-value	Correlation factor r^2					
Antioxidant activity EC_{50} values (mg/mL				**TPA**	**TED**	**TF3O**	**TDF**	**TPC**	**TA**
DPPH scavenging activity	164 ± 4	282 ± 7	<0.001	0.9855	0.9892	0.9876	0.9812	0.9897	0.9905
Reducing power	62.0 ± 0.1	185.0 ± 3.2	<0.001	0.9929	0.9967	0.995	0.9887	0.9972	0.998
β-carotene bleaching inhibition	28 ± 2	100 ± 6	<0.001	0.9891	0.9819	0.9818	0.9736	0.9825	0.9843
TBARS inhibition	9.2 ± 0.2	33 ± 1	<0.001	0.9916	0.9954	0.9937	0.9873	0.9958	0.9966
Antimicrobial activity MIC values (mg/mL)									
Gram negative bacteria									
Acinetobacter baumannii	4	4	-	-	-	-	-	-	-
Escherichia coli ESBL 1*	1 (47%**)	1	-	-	-	-	-	-	-
Escherichia coli ESBL 2*	0.25 (49%**)	0.25	-	-	-	-	-	-	-
Escherichia coli	0.5	2	-	0.9938	0.9976	0.9959	0.9895	0.998	0.9989
Klebsiella pneumoniae	1	1	-	-	-	-	-	-	-
Klebsiella pneumoniae ESBL*	1 (62%**)	1	-	-	-	-	-	-	-
Morganella morganii	2	2	-	-	-	-	-	-	-
Pseudomonas aeruginosa	2	4	-	0.9938	0.9976	0.9959	0.9895	0.998	0.9989
Gram positive bacteria									
Enterococcus faecalis	2	2	-	-	-	-	-	-	-
MRSA*	0.25 (85%**)	0.25	-	-	-	-	-	-	-
Streptococcus agalactae	1	1	-	-	-	-	-	-	-

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. MIC values correspond to the minimal sample concentration that inhibited the bacterial growth. TPA-Total phenolic acids; TED- Total ellagic acid derivatives; TF3O- Total flavan-3-ols; TF- Total flavonols; TPC- Total phenolic compounds (non-anthocyanins); TA- Total anthocyanins. * biofilm producers; ** inhibition percentage of biofilm production.

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3.2.3.4. References

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Supplemental material. Antibiotic susceptibility profile of Gram negative and Gram positive bacteria

	Gram negative								**Gram positive**		
	Escherichia coli	*Escherichia coli* ESBL 1*	*Escherichia coli* ESBL 2*	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* ESBL*	*Morganella morganii*	*Pseudomonas aeruginosa*	*Acinetobacter baumannii*	MRSA*	*Enterococcus faecalis*	*Streptococcus agalactiae*
Amikacin	na	S	R	na	S	na	S	S	na	na	na
Amoxicillin/Clavulanic acid	S	R	R	S	R	R	na	na	na	na	na
Ampicilin	R	R	na	R	R	R	na	na	na	S	S
Cefotaxime	na	R	na	R	R	na	na	R	na	na	na
Ceftalorina	na	na	na	na	na	na	na	na	na	na	na
Ceftazidime	na	R	R	na	R	R	R	R	na	na	na
Cefuroxime	S	R	na	R	R	na	na	na	na	na	na
Ciprofloxacin	R	R	S	R	R	R	R	na	na	na	na
Clindamycin	na	na	na	na	na	na	na	na	R	na	S
Colistin	na	na	S	na	na	na	S	na	na	na	na
Erythromycin	na	na	na	na	na	na	na	na	R	na	S
Ertapenem	na	S	na	S	S	S	na	na	na	na	na
Fosfomycin	S	na	na	S	na	na	na	na	R	na	na
Gentamicin	R	S	s	S	R	S	R	S	R	na	na
Imipenem	na	na	S	na	na	na	R	na	na	na	na
Levofloxacin	na	R	na	na	R	na	R	S	R	na	na
Linezolide	na	na	na	na	na	na	na	na	S	na	na
Meropenem	na	S	S	na	S	na	R	R	na	na	na
Minocycline	na	na	R	na	na	na	na	S	na	na	na
Nitrofurantoin	S	S	na	R	R	R	na	na	S	S	na
Norfloxacin	R	na	na	R	na	na	na	na	na	na	na
Oxacilin	na	na	na	na	na	na	na	na	R	na	na
Penicillin	na	na	na	na	na	na	na	na	na	na	na
Piperacillin/Tazobactam	na	S	R	S	R	R	R	R	na	na	na
Tobramycin	na	S	R	na	R	na	R	na	na	na	na
Trimethoprim/sulfamethoxazole	R	R	S	R	R	S	na	S	S	na	na
Vancomicin	na	na	na	na	na	na	na	na	S	na	na

ESBL- Extended spectrum betalactamase; S- susceptible; R-resistant (this classification was made according to the interpretive breakpoints suggested by the Clinical and Laboratory Standards Institute and CLSI European Committee on Antimicrobial Susceptibility Testing - EUCAST); na- not applicable; * biofilm producers.

3.3. Laurus nobilis L.

Neste sub-capítulo apresenta-se a caraterização nutricional e química, e as propriedades antioxidantes, citotóxicas e antimicrobianas de *Laurus nobilis* L. silvestre e comercial e das respetivas infusões e extratos metanol: água.

3.3.1. Contribuições nutricionais e antioxidantes de folhas de *Laurus nobilis* L.: seria mais adequado uma amostra silvestre ou cultivada?

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Nutritional and antioxidant contributions of Laurus nobilis L. leaves: would be more suitable a wild or a cultivated sample?.

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Abstract

Medicinal and aromatic plants are used since ancient times in folk medicine and traditional food, but also in novel pharmaceutical preparations. The controversy lies in the use of cultivated and/or wild plants presenting both advantages and disadvantages in biological, ecological but also economic terms. Herein, cultivated and wild samples of *Laurus nobilis* L. were chemically characterized regarding nutritional value, free sugars, organic acids, fatty acids and tocopherols. Furthermore, the antioxidant activity (scavenging activity, reducing power and lipid peroxidation inhibition) and individual phenolic profile of *L. nobilis* extracts and infusions were evaluated. Data showed that the wild sample gave higher nutritional contribution related to a higher content of proteins, free sugars, organic acids, PUFA and tocopherols. It also gave better PUFA/SFA and n-6/n-3 ratios. Regarding antioxidant activity and phenolic compounds, it was the cultivated sample (mostly the infusion) that showed the highest values. The present study supports the arguments

defending the use of wild and cultivated medicinal and aromatic plants as both present very interesting features, whether nutritional or antioxidant, that can be an assessed by their consumption. *In vitro* culture could be applied to *L. nobilis* as a production methodology that allows combination of the benefits of wild and cultivated samples.

Keywords: Laurus nobilis L.; Cultivated/Wild; Chemical characterization; Antioxidant properties; Phenolic profile

3.3.1.1. Introduction

Currently, there is a major controversy concerning the use of wild or cultivated plants, presenting both advantages and disadvantages in biological and ecological, but also economic terms (Schippmann, Leaman, & Cunningham, 2002). Due to the growing demand of global market, FAO (Food and Agricultural Organization) recommended the cultivation of medicinal and aromatic plants, not only from the point of view of sustainability but also because it allows better control of biotic and abiotic production conditions, representing a reliable resource of raw material that has gained great economic importance (Schippmann et al, 2002). Being used since ancient times for their organoleptic characteristics, therapeutic and medicinal properties, it is crucial to preserve the genetic-pool resources that these plants represent (Guarrera & Savo, 2013). On the other hand, the use of wild medicinal and aromatic plants by many local populations provides herbal medicines for health care needs encouraging their protection and maintenance, not requiring the use of pesticides neither investments in infrastructures to produce them (Schippmann et al, 2002).

Laurus nobilis L., commonly known as bay leaves, belongs to Laureacea family, being a native plant from the warm Mediterranean region, including countries like Italy, France, Spain and Portugal. It is widely used as a spicy fragrance and flavor in traditional meat dishes, stews and rice (Camejo-Rodrigues, Ascenção, Bonet, & Valles, 2003; Gómez-Coronado & Barbas, 2003; Ouchikh et al, 2011). Its leaves and extracts are used to suppress high blood sugar, fungal and bacterial infections, to treat eructation, flatulence and gastrointestinal problems. It also exhibits anti-inflammatory, anticonvulsive, antiepileptic and antioxidant properties (Ferreira, Proença, Serralheiro, & Araújo, 2006; Conforti, Statti, Uzunov, & Menichini, 2006; Ozcan, Esen, Sangun, Coleri, & Caliskan, 2010; Polovka & Suhaj, 2010; Ouchikh et al, 2011; Speroni et al, 2011; Ramos et al, 2012). Infusions of dry bay leaves are used in folk medicine for their stomachic and carminative remedies and also to treat gastric diseases (Afifi, Khalil, Tamimi, & Disi, 1997; Dall'Acqua et al, 2009).

Tocopherols content of *L. nobilis* was reported on aerial parts (Demo, Petrakis, Kefalas, & Boskou, 1998; Gómez-Coronado & Barbas, 2003; Gómez-Coronado, Ibañez,

Ruperéz, & Barbas, 2004) and vegetative organs (Ouchikh et al, 2011); fatty acids composition was studied on seeds (Ozcan et al., 2010).

Antioxidant activity of wild *L. nobilis* leaves was previously reported on ethanol and aqueous extracts (Elmastaş et al., 2006; Emam, Mohamed, Diab, & Megally, 2010; Kaurinovic, Popovic, & Vlaisavljevic, 2010; Ramos et al., 2012), methanol/water extracts (Conforti et al., 2006) and infusions (Dall'Acqua et al., 2009). Flavonoids such as quercetin, luteolin, apigenin, kaempferol and myrcetin derivatives as well as flavan-3-ols have been reported as the most abundant phenolic compounds found in bay leaves (Škerget et al, 2005; Dall'Acqua et al., 2009; Lu, Yuan, Zeng, & Chen, 2011). The hydroxyl groups attached to the ring structure of flavonoids conferred them antioxidant properties, acting as reducing agents, hydrogen donators, metal chelators and radical scavengers, preventing oxidative stress, the main cause of cell death (Carocho & Ferreira, 2013).

In the present work, *L. nobilis* wild and cultivated samples were chemically characterized regarding nutritional value, free sugars, organic acids, fatty acids and tocopherols. Furthermore, as far as we know, this is the first study comparing antioxidant activity and phenolic compounds of extracts and infusions of *L. nobilis* cultivated and wild samples.

3.3.1.2. Materials and methods

Samples

The cultivated air-dried *Laurus nobilis* L. sample (leaves) was purchased from a local company, Ervital from Castro Daire, Portugal, which produces Mediterranean herbs using organic principles and methods. The wild sample (leaves) was collected in the fall on Bragança, Portugal, and further lyophilized (FreeZone 4.5, Labconco, Kansas, USA).

Each sample was reduced to a fine dried powder (20 mesh) and mixed to obtain homogenate sample.

Standards and Reagents

Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and the fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also were other individual fatty acid isomers, L-ascorbic acid, tocopherol, sugar and organic acid standards. Phenolic compound standards were from Extrasynthese (Genay, France). Racemic tocol, 50 mg/mL, was purchased from Matreya (Pleasant Gap, PA USA). 2,2-Diphenyl-1-

picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Nutritional contribution of wild and cultivated samples

Proximate composition and energetic value. The samples were analysed for proteins, fat, carbohydrates and ash using the AOAC procedures (AOAC, 1995). The crude protein content (N×6.25) of the samples was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600±15 ^{o}C. Total carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein) + 3.75 × (g carbohydrate) + 9 × (g fat).

Sugars. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI), after an extraction procedure previously described by the authors (Guimarães et al, 2013) using melezitose as internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco) and an RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6×250 mm, 5 mm, Knauer) operating at 30 ºC (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards analysed in the same conditions. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight.

Organic acids. Organic acids were determined following a procedure previously described by the authors (Pereira, Barros, Carvalho, & Ferreira, 2013). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation). Separation was achieved on a SphereClone (Phenomenex) reverse phase C_{18} column (5 μm, 250 mm × 4.6 mm i.d.) thermostatted at 35 ºC. The elution was performed with sulphuric acid 3.6 mM using a flow rate of 0.8 mL/min. Detection was carried out in a PDA, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

Fatty acids. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID)/capillary column as described previously by the authors (Guimarães et al, 2013). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 ºC) and a Macherey–Nagel column (30 m × 0.32 mm × 0.25 µm). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30 ºC/min ramp to 125 ºC, 5 ºC/min ramp to 160 ºC, 20 ºC/ min ramp to 180 ºC, 3 ºC/min ramp to 200 ºC, 20 ºC/min ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with those of standards. The results were recorded and processed using the CSW 1.7 Software (DataApex 1.7) and expressed in relative percentage of each fatty acid.

Tocopherols. Tocopherols were determined following a procedure previously described by the authors (Guimarães et al, 2013). Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II (250 mm × 4.6 mm i.d.) normal-phase column from YMC Waters operating at 30 ºC. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min, and the injection volume was 20 µL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry weight.

Antioxidants contribution of wild and cultivated samples

Methanolic extract and infusion preparations. The methanolic extract was obtained from the wild and cultivated plant material. Each sample (1 g) was extracted twice by stirring with 30 mL of methanol (25 ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper (Guimarães et al, 2013). The combined methanolic extracts were evaporated at 40 ºC (rotary evaporator Büchi R-210) to dryness.

For infusion preparation the plant material (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure (Guimarães et al, 2013). The obtained infusion was frozen and lyophilized.

Antioxidant activity evaluation. Methanolic extracts and lyophilized infusions were redissolved in methanol and water, respectively (final concentration 2.5 mg/mL) for antioxidant activity evaluation. The final solutions were further diluted to different concentrations to be submitted to the following assays. DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Guimarães et al, 2013). The final results were expressed in EC_{50} values (mg/mL), sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay). Trolox was used as positive control.

Phenolic profile. Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) as previously described by the authors (Rodrigues et al, 2012). Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared from different standard compounds: catechin (y=158.42x+11.38; R^2=0.999); epicatechin (y=129.11x+11.663, R^2=0.9999); rutin (y=281.98x-0.3458; R^2=1); kaempferol-3-*O*-glucoside (y=288.55x-4.05; R^2=1); kaempferol-3-*O*-rutinoside (y=239.16x-10.587; R^2=1); apigenin-6-*C*-glucoside (y=223.22x+60.915, R^2=1); luteolin-6-*C*-glucoside (y=508.54x-152.82; R^2=0.997); luteolin-7-*O*-glucoside (y=80.829x-21.291; R^2=0.999); quercetin-3-*O*-glucoside (y=253.52x-11.615; R^2=0.999) and isorahmetin-3-*O*-rutinoside (y=327.42x+313.78; R^2=0.999) The results were expressed in mg per g of methanolic extract and lyophilized infusion.

Statistical analysis

For wild and cultivated plant material, three samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with α = 0.05. This treatment was carried out using SPSS v. 18.0 program.

3.3.1.3. Results and Discussion

Nutritional contribution of wild and commercial samples

Data on the chemical composition of cultivated and wild samples of *L. nobilis* namely, macronutrients, sugars and organic acids are presented in **Table 25**. Carbohydrates (including fiber) were the major macronutrients found in both samples, followed by proteins, fat and ash. Both samples revealed similar contents of carbohydrates, fat, ash and energetic values whereas the wild sample showed higher protein contents.

Table 25. Macronutrients, free sugars and organic acids of cultivated and wild *Laurus nobilis*.

	Cultivated	**Wild**
Fat (g/100 g dw)	5.47 ± 0.00[a]	5.41 ± 0.00[a]
Proteins (g/100 g dw)	10.44 ± 0.02[b]	13.24 ± 0.03[a]
Ash (g/100 g dw)	4.83 ± 0.05[a]	5.09 ± 0.41[a]
Carbohydrates (g/100 g dw)	79.27 ± 0.03[a]	76.26 ± 0.31[a]
Energy (kcal/100 g dw)	408.06 ± 0.14[a]	406.69 ± 1.16[a]
Fructose	1.68 ± 0.02[a]	1.40 ± 0.12[b]
Glucose	1.17 ± 0.17[b]	1.78 ± 0.32[a]
Sucrose	1.34 ± 0.10[b]	2.60 ± 0.61[a]
Total sugars (g/100 g dw)	4.19 ± 0.09[b]	5.79 ± 0.41[a]
Oxalic acid	0.43 ± 0.01[b]	0.55 ± 0.00[a]
Malic acid	0.25 ± 0.03[a]	0.35 ± 0.00[a]
Ascorbic acid	nd	0.03 ± 0.00
Total organic acids (g/100g dw)	0.68 ± 0.02[a]	0.90 ± 0.01[a]

nd- not detected; dw- dry weight. In each row different letters mean significant differences (p>0.05).

Fructose, glucose and sucrose were the free sugars detected in the studied samples. The wild sample gave the highest contents in total free sugars and also in sucrose and glucose. Fructose was the major free sugar found in the cultivated sample. The wild sample also revealed the highest content of organic acids. Oxalic and malic acids were found in both samples, but ascorbic acid was only found in wild bay leaves (**Table 25**). The several processes applied to cultivated samples throughout the supply chain (preharvest conditions, postharvest handling, storage conditions, processing, and preparation) could contribute to degradation of ascorbic acid, which is a vitamin susceptible to degradation in non-fresh samples, but the maturity at harvest and the genetic variations that both samples presente

could also influence the differences found on ascorbic acid cocnentration (Howard, Wong, Peery, & Klein, 1999).

Up to twenty-five fatty acids were found in cultivated and wild samples of *L. nobilis* (**Table 26**).

Table 26. Fatty acids and tocopherols of cultivated and wild *Laurus nobilis*.

Fatty acid	Cultivated	Wild
C6:0	0.64 ± 0.01^{a}	0.45 ± 0.10^{b}
C8:0	0.37 ± 0.03^{a}	0.08 ± 0.01^{b}
C10:0	0.35 ± 0.05^{a}	0.29 ± 0.08^{b}
C12:0	1.73 ± 0.08^{a}	0.54 ± 0.14^{b}
C13:0	2.46 ± 0.15^{a}	1.36 ± 0.37^{b}
C14:0	5.27 ± 0.05^{a}	1.31 ± 0.22^{b}
C14:1	0.60 ± 0.03^{a}	0.41 ± 0.07^{b}
C15:0	0.95 ± 0.02^{a}	0.36 ± 0.11^{b}
C15:1CIS-10	0.17 ± 0.01^{a}	0.15 ± 0.04^{a}
C16:0	25.97 ± 0.25^{a}	13.47 ± 0.57^{b}
C16:1	0.58 ± 0.07^{a}	0.50 ± 0.10^{a}
C17:0	1.32 ± 0.00^{a}	0.62 ± 0.00^{b}
C17:1CIS-10	0.13 ± 0.01^{b}	0.29 ± 0.02^{a}
C18:0	8.77 ± 0.12^{a}	3.39 ± 0.01^{b}
C18:1n9	9.00 ± 0.01^{a}	3.78 ± 0.36^{b}
C18:2n6	9.64 ± 0.10^{b}	12.40 ± 0.51^{a}
C18:3n6	0.42 ± 0.11^{a}	0.20 ± 0.13^{b}
C18:3n3	13.40 ± 0.07^{b}	51.59 ± 1.12^{a}
C20:0	1.57 ± 0.02^{a}	1.11 ± 0.00^{b}
C20:1CIS-11	0.38 ± 0.04^{a}	0.15 ± 0.04^{b}
C20:3n3+C21:0	0.54 ± 0.07^{a}	0.32 ± 0.01^{b}
C22:0	2.58 ± 0.05^{a}	1.06 ± 0.00^{b}
C23:0	1.18 ± 0.02^{a}	0.44 ± 0.01^{b}
C24:0	11.96 ± 0.03^{a}	5.71 ± 0.31^{b}
SFA	65.11 ± 0.10^{a}	30.23 ± 1.92^{b}
MUFA	10.70 ± 0.10^{a}	5.12 ± 0.20^{b}
PUFA	24.01 ± 0.01^{b}	64.50 ± 1.76^{a}
PUFA/SFA	0.37 ± 0.02^{b}	2.14 ± 0.14^{a}
n6/n3	0.72 ± 0.00^{a}	0.24 ± 0.01^{b}
α - tocopherol	304.74 ± 16.89^{b}	370.05 ± 0.56^{a}
β - tocopherol	45.14 ± 0.77^{a}	13.53 ± 0.15^{b}
γ - tocopherol	302.33 ± 6.47^{b}	395.76 ± 2.64^{a}
δ - tocopherol	3.49 ± 0.02^{a}	0.78 ± 0.12^{b}
Total tocopherols (mg/100 g dw)	655.70 ± 22.62^{b}	780.12 ± 2.36^{a}

nd- not detected; dw- dry weight. Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0); Tridecanoic acid (C13:0); Myristic acid (C14:0); Myristoleic acid (C14:1); Pentadecanoic acid (C15:0); cis-10-Pentadecenoic acid (C15:1); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0) cis-10-Heptadecenoic acid (C17:1); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6c); □-Linolenic acid (C18:3n6); Linolenic acid (C18:3n3); Arachidic acid (C20:0); cis-11-Eicosenoic acid (C20:1); cis-11,14,17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3+C21:0); Behenic acid (C22:0); Tricosanoic acid (C23:0); Lignoceric acid (C24:0). SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids. In each row different letters mean significant differences between species (p□0.05).

Palmitic acid (C16:0; SFA) was the major fatty acid present in the cultivated sample, followed by linolenic acid (C18:3n-3; PUFA), while in the cultivated sample the opposite was observed. Thus, the highest levels of saturated fatty acids (SFA) were found in the cultivated sample, while wild bay leaves gave the highest content of polyunsaturated fatty acids

(PUFA). Ozcan et al. (2010) reported linoleic and lauric acids as the main fatty acids in *L. nobilis* seeds, followed by palmitic acid. For a "good nutritional quality" with high health benefits, ratio on PUFA/SFA should be higher than 0.45 and n-6/n-3 fatty acids should be lower than 4.0 (Guil et al, 1996). Both samples presented the required values, however wild sample of bay leaves presented a higher value of PUFA/SFA ratio and a lower value of n-6/n-3 fatty acids ratio. All the isoforms of tocopherols were found in both samples of bay leaves (**Table 26**). Once more, the wild sample showed the highest total tocopherols content, mainly γ-tocopherol followed by α-tocopherol. Previous studies conducted using different extraction methodologies including a saponification step (Demo et al., 1998; Ouchikh et al., 2011) and supercritical fluids (Gómez-Coronado, 2004), or even different extraction solvents (Gómez-Coronado & Barbas, 2003), reported much lower tocopherols content and not detecting all the isoforms reported herein.

Antioxidants contribution of wild and commercial samples

The antioxidant activity of methanolic extract and infusion of cultivated and wild *L. nobilis* was studied and the results are presented in **Table 27**. Both preparations were chosen because infusions and extracts of the leaves are widely used in medicinal practices, as stated in the introduction section. In general, infusions of both samples revealed higher antioxidant activity (lower EC_{50} values) than methanolic extracts. Cultivated *L. nobilis* showed higher DPPH scavenging activity, reducing power and TBARS inhibition than the wild sample. The samples studied herein showed higher DPPH scavenging activity than the aqueous-methanol and aqueous extracts of *L. nobilis* from Finland (EC_{50}=0.55 mg/mL; Koşar, Dorman, & Hiltunen, 2005) and Montenegro (EC_{50}=0.16 mg/mL; Kaurinovic et al., 2010). Santoyo et al (2006) showed that, in supercritical extraction fluids, the antioxidant activity of *L. nobilis* increases, with lower EC_{50} values for DPPH (EC_{50}=0.10 mg/mL) and β-carotene (EC_{50}=0.04 mg/mL) assays. As stated by Papageorgiou, Mallouchos, & Komaitis (2008), the use of different drying methods influences the antioxidant activity of bay leaves. Finally, Conforti et al. (2006) described the wild sample (but ethanolic extracts) as having higher antioxidant activity than cultivated bay leaves.

Table 27. Antioxidant activity of methanolic extracts and infusions of cultivated and wild *Laurus nobilis*.

	Cultivated		Wild	
	Methanolic extract	**Infusion**	**Methanolic extract**	**Infusion**
DPPH scavenging activity (EC_{50}, mg/mL)	0.15 ± 0.00^{b}	0.09 ± 0.00^{d}	0.20 ± 0.00^{a}	0.13 ± 0.01^{c}
Reducing power (EC_{50}, mg/mL)	0.12 ± 0.00^{b}	0.09 ± 0.00^{c}	0.14 ± 0.00^{a}	0.12 ± 0.00^{b}
β-carotene bleaching inhibition (EC_{50}, mg/mL)	0.18 ± 0.02^{a}	0.16 ± 0.02^{a}	0.10 ±0.01^{b}	0.20 ± 0.03^{a}
TBARS inhibition (EC_{50}, mg/mL)	0.01 ± 0.00^{b}	0.01 ± 0.00^{b}	0.03 ± 0.00^{a}	0.02 ± 0.01^{b}

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. In each row different letters mean significant differences ($p<0.05$).

The HPLC phenolic profile of a wild sample of *L. nobilis* recorded at 280 and 370 nm is shown in **Figure 15** and Erro! A origem da referência não foi encontrada., respectively, and peak characteristics and tentative identities are presented in **Table 28**. Thirty-two compounds were detected, from which fourteen were flavan-3-ols (i.e., catechins and proanthocyanidins), fourteen flavonols and four flavones.

Flavan-3-ols, peaks 1-13 and 16, were identified according to their UV spectra and pseudomolecular ions. Peaks 3, 5 and 8 were identified as (+)-gallocatechin, (+)-catechin and (-)-epicatechin, respectively, by comparison of their UV spectra and retention time with authentic standards. Peaks 1 and 2 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 451, releasing an MS^2 fragment at *m/z* 289 ($[M-H-162]^-$, loss of an hexosyl moiety), corresponding to a catechin monomer. These compounds were tentatively identified as (epi)catechin hexosides, identity that was coherent with their earlier elution (higher polarity) compared with the parent aglycones. Other signals at *m/z* 577, 865 and 1153 (peaks 4, 6, 7, 11-13 and 16), can be respectively associated to B-type procyanidin dimers, trimers and tetramers (*i.e.*, (epi)catechin units with C4-C8 or C4-C6 interflavonoid linkages). Furthermore, peaks 9 and 10 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 863 that could correspond to a procyanidin trimer containing one B-type and one A-type (i.e., C4-C8 or C4-C6 and C2-*O*-C7 or C2-*O*-C5) interflavonoid linkages.

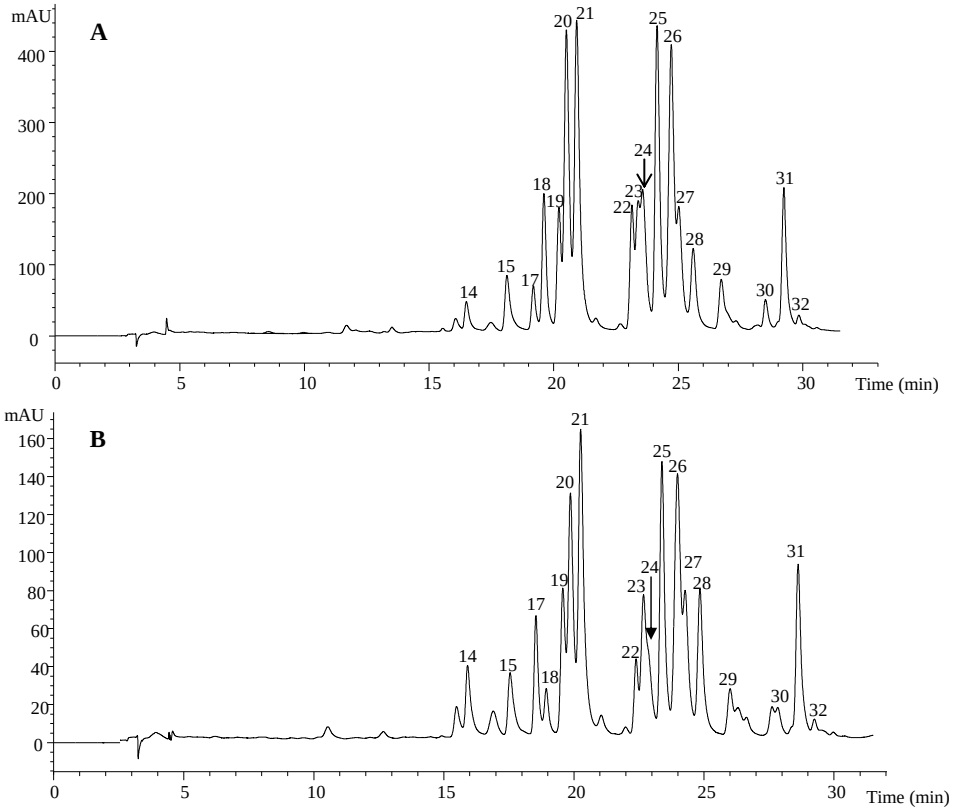

Figure 15. HPLC phenolic profile (flavone/ols) of cultivated (**A**) and wild (**B**) *Laurus nobilis*, obtained at 370 nm. Identification of peaks 14, 15 and 17–32 is presented in **Table 28**.

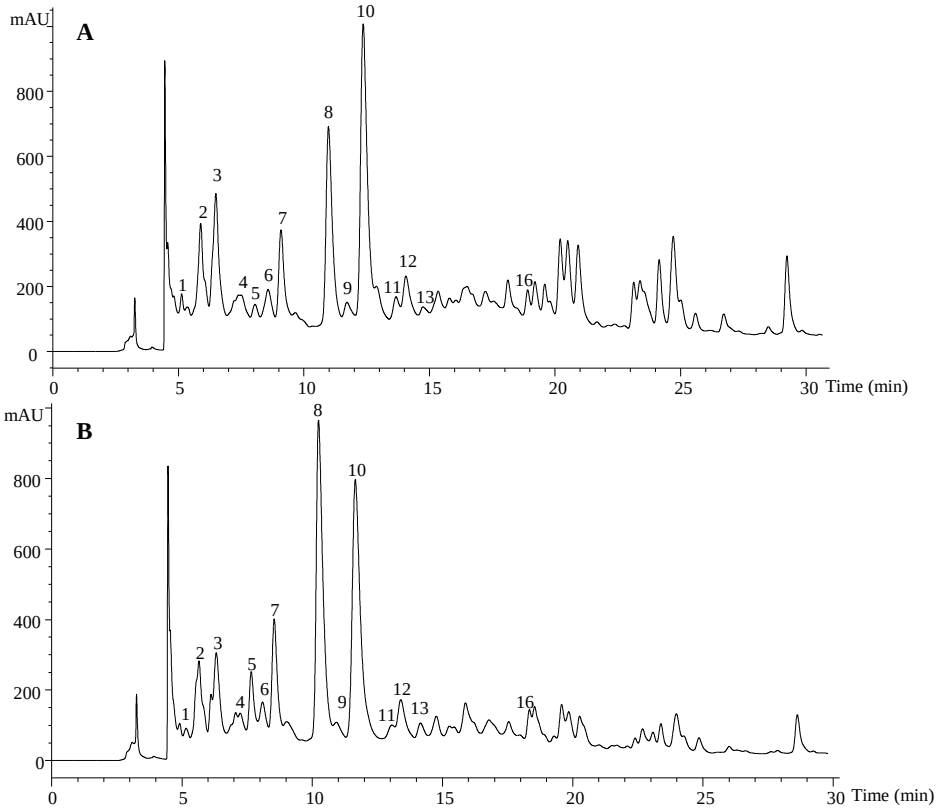

Figure 16. HPLC phenolic profile (flavan-3-ols) of cultivated (**A**) and wild (**B**) *Laurus nobilis*, obtained at 280 nm. Identification of peaks 1–13 and 16 is presented in **Table 28**.

Table 28. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification of flavonoids in *Laurus nobilis*.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	5.12	278	451	289(100)	(Epi)catechin-hexoside
2	5.88	278	451	289(100)	(Epi)catechin-hexoside
3	6.49	276	305	219(13),179(24),125(10)	(+)-Gallocatechin
4	7.49	278	1151	865(11), 713(16), 577(7),575(35),561(5), 289(44)	Procyanidin tetramer
5	8.05	279	289	245(79), 203(58), 137(24)	(+)-Catechin
6	8.57	280	577	451(28), 425(60), 407(83), 289(61), 287(13)	Procyanidin dimer
7	9.08	279	577	451(49), 425(82), 407(100), 289(69), 287(15)	Procyanidin dimer
8	10.97	278	289	245 (83), 205(46), 151(24), 137(26)	(-)-Epicatechin
9	11.71	276	863	711(53), 573(27), 451(30), 411(43), 289(22), 285(9)	Procyanidin trimer (B- and A-type linkages)
10	12.36	278	863	711(46), 573(27), 451(34), 411(46), 289(20), 285(8)	Procyanidin trimer (B- and A-type linkages)
11	13.66	280	1153	865(9), 713(4), 577(29),575(14),561(6), 289(23)	Procyanidin tetramer
12	14.06	280	1153	865(13), 713(9), 577(33),575(30),561(5), 289(61)	Procyanidin tetramer
13	14.74	280	865	739(8),713(17), 695(9), 577(16), 575(25), 425(8),407(16), 289(7), 287(15)	Procyanidin trimer
14	16.50	350	447	357(72), 327(74), 297(14)	Luteolin 6-*C*-glucoside
15	18.12	337	431	341(16),311(100)	Apigenin 8-*C*-glucoside
16	18.91	280	577	451(49), 425(85), 407(97), 289(89), 287(22)	Procyanidin dimer
17	19.18	338	577	457(8),413(49),341(7),311(6),293(34)	2''-*O*-Rhamnosyl-*C*-hexosyl-apigenin
18	19.59	355	609	301(100)	Quercetin 3-*O*-rutinoside
19	20.21	336	431	341(76),311(100)	Apigenin 6-*C*-glucoside
20	20.51	356	463	301(100)	Quercetin 3-*O*-glucoside
21	20.92	355	463	301(100)	Quercetin *O*-hexoside
22	23.14	347	593	285(100)	Kaempferol 3-*O*-rutinoside
23	23.36	344	433	301(100)	Quercetin *O*-pentoside
24	23.56	350	447	285(100)	Kaempferol 3-*O*-glucoside
25	24.15	354	623	315(100)	Isorhamnetin *O*-rutinoside
26	24.71	348	447	301(100)	Quercetin *O*-rhamnoside
27	25.01	356	477	315(100)	Isorhamnetin *O*-hexoside
28	25.60	354	477	315(100)	Isorhamnetin *O*-hexoside
29	26.72	347	417	285(100)	Kaempferol *O*-pentoside
30	28.49	355	447	315(100)	Isorhamnetin *O*-pentoside
31	29.23	343	431	285(100)	Kaempferol *O*-hexoside
32	29.85	350	461	315(100)	Isorhamnetin *O*-rhamnoside

Fourteen flavonols derivatives were also detected, five of them derived from quercetin (λ_{max} around 350 nm and an MS^2 fragment at *m/z* 301), other five from isorhamnetin (λ_{max} around 354 nm and an MS^2 fragment at *m/z* 315) and four from kaempferol (λ_{max} around 347 nm and an MS^2 fragment at *m/z* 285) (**Table 28**). Quercetin 3-*O*-rutinoside (peak 18), quercetin 3-*O*-glucoside (peak 20), kaempferol 3-*O*-rutinoside (peak 22), kaempferol 3-*O*-glucoside (peak 24) and isorhamnetin 3-*O*-rutinoside (peak 25) were positively identified according to their retention, mass and UV-vis characteristics by comparison with a commercial standard.

Peaks 21, 23 and 26 ($[M-H]^-$ at *m/z* 463, 433 and 447, respectively) were assigned to quercetin (*m/z* at 301) derivatives; peaks 29 and 31 ($[M-H]^-$ at *m/z* 417 and 431, respectively) were assigned to kaempferol (*m/z* at 285) derivatives and peaks 27, 28, 30 and 32 ($[M-H]^-$ at *m/z* 477, 447 and 461, respectively) were assigned to isorhamnetin (*m/z* at 315) derivatives, presenting distinct losses of hexosyl (-162 mu), pentosyl (-132 mu) and rhamnosyl (-146 mu) moieties. Their elution order was coherent with the type of substituent sugars, according to their expected polarity, although the position and nature of the sugar moieties could not be identified, because their retention times did not correspond to any of the standards available.

The remaining phenolic compounds corresponded to *C*-glycosylated flavones, three apigenin derivatives (peaks 15, 17 and 19) and one luteolin derivative (peak 14), according to their UV spectra (λ_{max} around 337 for apigenin and 350 nm for luteolin) and MS^2 fragmentation pattern (**Table 28**). Peaks 15 and 19 showed the same pseudomolecular ion $[M-H]^-$ at *m/z* 431 giving place to two MS^2 fragment ions, a major one at *m/z* 341 $[M-90]^-$, and another one at *m/z* 311 $[M-120]^-$. This fragmentation pattern was characteristic of *C*-glycosylated flavones at C-6/C-8, and the relative abundance of fragments pointed out to sugar substitution at C-8 (peak 15) at C-6 (peak 19) according to the fragmentation patterns described by Ferreres, Silva, Andrade, Seabra, & Ferreira (2003) and Ferreres, Llorach, & Gil-Izquierdo (2004). These peaks were respectively identified as apigenin 8-*C*-glucoside and apigenin 6-*C*-glucoside; the identity of this latter was further confirmed by comparison with an authentic standard. Peak 17 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 577, releasing typical MS^2 fragments ions. The loss of 120 mu (ion at *m/z* 457 ($[M-H-120]^-$) is characteristic of *C*-hexosyl flavones (Ferreres et al., 2003), while the loss of 164 mu, releasing the fragment at *m/z* 413 ($[M-H-146-18]^-$) can be associated to an *O*-glycosylation on the hydroxyl group at position 2 of the *C*-glycosylating sugar (Ferreres, Gil-Izquierdo, Andrade, Valentão & Tomás-Barberán, 2007). The remaining ions at *m/z* 341 ($[aglycone + 71)]^-$, *m/z* 311 ($[aglycone + 41)]^-$ and *m/z* 293 ($[aglycone + 41-18]^-$) are usual in mono-*C*-glycosyl derivatives

O-glycosylated on 2'' position (Ferreres et al., 2007). According to this fragmentation pattern the compound was tentatively identified as 2''-*O*-rhamnosyl-*C*-hexosyl-apigenin.

Peak 14 was assigned to a luteolin derivative. It showed a pseudomolecular ion $[M-H]^-$ at *m/z* 447 giving place to three MS^2 fragment ions, a major one at *m/z* 357 $[M-H-90]^-$, and other two at *m/z* 327 $[M-H-120]^-$ and at *m/z* 297 $[M-H-30]^-$. This fragmentation pattern and the relative abundance of fragments was characteristic of *C*-glycosylated flavones at C-6 (Ferreres et al., 2003, 2004). The peak was identified as luteolin-6-*C*-glucoside, which was further confirmed by comparison to a standard.

The cultivated sample presented higher concentration of phenolic compounds, especially flavonol and flavone derivatives, when compared to the wild sample; on the other hand, the flavan-3-ols concentration was very similar in both types of samples. Flavan-3-ols were the major phenolic compounds present in both wild and commercial samples (**Table 29**), being (-)-epicatechin and a procyanidin trimer with an A-type linkage the most abundant ones. Škerget et al. (2005) reported the identification of flavonols such as quercetin and kaempferol derivatives and flavan-3-ols in the methanolic extract of *L. nobilis* from Slovenia, but in much lower concentrations than in our samples. Dall'acqua et al. (2009) identified ten major peaks in the infusion of *L. nobilis* from Italy corresponding to kaempferol and quercetin glycosides derivatives and flavan-3-ols (mainly catechin and proanthocyanidins), although these latter in very low amounts. Lu et al. (2011) reported the presence of flavonoids and low concentrations of phenolic acids in ethanolic extracts of *L. nobilis* from China, but with a single identification of rutin; all the phenolic acids were indicated as unknown. No relevant amounts of phenolic acid derivatives were detected in the samples here analysed.

Table 29. Concentrations of phenolic compounds (mg/g of methanolic extract or infusion) in wild and cultivated *Laurus nobilis*.

	Cultivated		Wild	
Phenolic compounds	**Methanolic extract**	**Infusion**	**Methanolic extract**	**Infusion**
(Epi)catechin-hexoside	0.55 ± 0.12	0.51 ± 0.02	0.34 ± 0.03	0.68 ± 0.06
(Epi)catechin-hexoside	3.92 ± 0.14	3.36 ± 0.33	2.17 ± 0.09	4.05 ± 0.41
(+)-Gallocatechin	5.97 ± 0.10	4.20 ± 0.27	3.79 ± 0.12	3.44 ± 0.31
Procyanidin tetramer	0.78 ± 0.07	0.82 ± 0.03	1.09 ± 0.12	0.79 ± 0.18
(+)-Catechin	0.76 ± 0.02	0.87 ± 0.05	2.88 ± 0.02	3.66 ± 0.22
Procyanidin dimer	1.92 ± 0.04	1.22 ± 0.23	1.82 ± 0.15	1.21 ± 0.10
Procyanidin dimer	4.68 ± 0.12	3.78 ± 0.18	5.41 ± 0.13	5.59 ± 0.44
(-)-Epicatechin	15.69 ± 0.62	12.35 ± 0.43	22.18 ± 0.83	23.08 ± 0.45
Procyanidin trimer (B- and A-type linkages)	1.25 ± 0.09	0.72 ± 0.07	1.11 ± 0.00	0.60 ± 0.00
Procyanidin trimer (B- and A-type linkages)	20.19 ± 0.21	13.91 ± 0.31	17.83 ± 0.18	9.66 ± 0.04
Procyanidin tetramer	1.75 ± 0.07	1.33 ± 0.19	0.82 ± 0.05	0.91 ± 0.01
Procyanidin tetramer	3.54 ± 0.23	2.52 ± 0.15	2.55 ± 0.03	1.78 ± 0.03
Procyanidin trimer	1.29 ± 0.08	0.85 ± 0.03	0.80 ± 0.06	0.73 ± 0.04
Luteolin 6-*C*-glucoside	1.35 ± 0.07	1.14 ± 0.07	1.29 ± 0.06	0.92 ± 0.00
Apigenin 8-*C*-glucoside	0.99 ± 0.01	0.97 ± 0.01	0.41 ± 0.01	0.32 ± 0.00
Procyanidin dimer	1.00 ± 0.03	0.74 ± 0.05	1.19 ± 0.08	0.69 ± 0.00
2''-*O*-Rhamnosyl-*C*-hexosyl-apigenin	0.56 ± 0.04	0.64 ± 0.03	0.55 ± 0.00	0.55 ± 0.00
Quercetin 3-*O*-rutinoside	1.58 ± 0.04	1.55 ± 0.06	0.21 ± 0.02	0.18 ± 0.01
Apigenin 6-*C*-glucoside	1.61 ± 0.05	1.44 ± 0.07	0.71 ± 0.02	0.48 ± 0.01
Quercetin 3-*O*-glucoside	4.32 ± 0.02	3.59 ± 0.05	1.29 ± 0.03	0.76 ± 0.03
Quercetin *O*-hexoside	4.99 ± 0.07	3.95 ± 0.10	1.76 ± 0.04	1.15 ± 0.04
Kaempferol 3-*O*-rutinoside	1.63 ± 0.03	1.58 ± 0.06	0.36 ± 0.00	0.34 ± 0.00
Quercetin *O*-pentoside	1.56 ± 0.24	1.38 ± 0.02	0.69 ± 0.04	0.41 ± 0.00
Kaempferol 3-*O*-glucoside	1.89 ± 0.16	1.45 ± 0.10	0.38 ± 0.04	0.19 ± 0.01
Isorhamnetin *O*-rutinoside	3.13 ± 0.05	3.02 ± 0.06	0.89 ± 0.00	0.71 ± 0.02
Quercetin *O*-rhamnoside	4.62 ± 0.09	3.85 ± 0.16	1.62 ± 0.00	1.10 ± 0.02
Isorhamnetin *O*-hexoside	1.29 ± 0.02	0.88 ± 0.03	0.44 ± 0.01	0.20 ± 0.01
Isorhamnetin *O*-hexoside	0.92 ± 0.06	0.59 ± 0.05	0.51 ± 0.01	0.27 ± 0.02
Kaempferol *O*-pentoside	0.67 ± 0.03	0.52 ± 0.02	0.24 ± 0.00	0.12 ± 0.00
Isorhamnetin *O*-pentoside	0.22 ± 0.05	0.13 ± 0.01	tr	tr
Kaempferol *O*-hexoside	1.83 ± 0.04	1.42 ± 0.03	0.81 ± 0.00	0.49 ± 0.01
Isorhamnetin *O*-rhamnoside	0.03 ± 0.01	tr	tr	tr
Total flavan-3-ols	**63.30 ± 0.21a**	**47.18 ± 1.79c**	**63.99 ± 0.43a**	**56.87 ± 2.07b**
Total flavonols	**28.69 ± 0.52a**	**23.91 ± 0.65b**	**9.20 ± 0.04c**	**5.64 ± 0.07d**
Total flavones	**4.52 ± 0.03a**	**4.19 ± 0.19b**	**2.96 ± 0.05c**	**2.26 ± 0.01d**
Total Phenolic compounds	**96.50 ± 0.77a**	**75.28 ± 2.64b**	**76.16 ± 0.34b**	**64.77 ± 2.14c**

Overall, the wild sample showed the highest content of proteins, free sugars, organic acids, PUFA and tocopherols. It also gave better PUFA/SFA and n-6/n-3 ratios. Regarding antioxidant activity and phenolic compounds, it was the cultivated sample (mostly the infusion) that showed the highest values. The present study supports the arguments defending the use of wild and cultivated medicinal and aromatic plants as both present interesting nutraceutical features: the wild sample gave higher nutritional contribution, but it was the cultivated sample that showed higher bioactivity. *In vitro* culture could be applied to *L. nobilis* as a production methodology that allows combination of the benefits of wild and cultivated samples.

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3.3.1.4. References

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3.3.2. Uma análise de componentes principais diferencia as atividades antitumorais e antimicrobianas de extratos metanol:água e aquosos de *Laurus nobilis* L. de diferentes origens

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Two-dimensional PCA highlights the differentiated antitumor and antimicrobial activity of hydromethanolic and aqueous extracts of Laurus nobilis L. from different origins

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Abstract

Natural matrices are crucial to find new and potent antitumor and antimicrobial compounds. *Laurus nobilis* L. (bay leaves), one of the most used culinary spices, could be a good candidate for that purpose, considering also its medicinal properties. Herein, *in vitro* antitumor (against five different human tumor cell lines) and antimicrobial (antibacterial and antifungal) activities of enriched phenolic extracts (obtained using different solvents, methanol and water) of *L. nobilis* from different origins (wild and cultivated), were evaluated together with phenolic compound groups. Principal component analysis (PCA) was applied in order to understand how each extract act differentially towards specific bacterial and fungal species, and also selected human tumor cell lines. The extract type induced the most marked changes in bioactivity of laurel samples. From the PCA biplot, it became clear that

wild bay leaves samples were higher inhibitors of tumor cell lines, especially HeLa, MCF7, NCI-H460 and HCT15. HepG2 had the same response to bay leaves from wild and cultivated origin. It was also observed that methanolic extracts tended to have higher antimicrobial activity, except *A. niger*, *A. fumigatus* and *P. verrucosum.* The differences in bioactivity might be related to the higher phenolic compounds contents presented by methanolic extracts. From the obtained results it is possible to choose the combination extract type/origin with potentially highest effect against determined bacteria, fungi or tumor cell line.

Keywords: Laurus nobilis L.; Cultivated/Wild; Antitumor; Antimicrobial; Principal Component Analysis.

3.3.2.1. Introduction

Laurus nobilis L. (Laureaceae), commonly known as bay leaves, is a native plant from the Southern Mediterranean region, often found in warm climate regions with high rainfall (Marzouki et al., 2013). It is one of the most widely used culinary spices for seasoning of meat products, soups and fishes, but also as an ornamental plant especially in Europe and USA, being also grown commercially in Turkey, Algeria, Morocco, Portugal, Spain, Italy, France and Mexico (Fang et al., 2005; Barla et al., 2007; Ivanoić et al., 2010). The dry bay leaves and their infusions are traditionally used to treat some gastrointestinal problems, such as epigastric, bloating, digestion, eructation and flatulence. It also possesses anticonvulsive and antiepileptic activities, and stimulant and narcotic properties (Barla et al., 2007; Panza et al., 2011; Dall'Acqua et al., 2009). The ability to supress high blood sugar, prevent migraines and headaches, but also bacterial and fungal infections, has also been reported (Fang et al., 2005; Ramos et al., 2013).

Natural matrices, like *L. nobilis*, are rich sources of bioactive compounds, being estimated that near 60% of the antitumor and anti-infectious drugs available on the market, or under clinical trial, are of natural origin (Al-Kalaldeh et al., 2011; Panza et al., 2011). The various biological activities of plant extracts are well recognized, namely their antifungal, antimicrobial, insecticidal and cytostatic effects; accordingly, the bioactivity of plant extracts is often explored in a multifactorial manner (Al-Kalaldeh et al., 2011; Dadalioğlu et al., 2004).

Nowadays, there is a worldwide concern about the use of synthetic chemical compounds as antitumor agents due to their potential negative health effects, opening ways to use plants as sources of natural compounds with similar activity (Carocho and Ferreira, 2013). On the other hand, the indiscriminate use of antibiotics to treat bacterial and fungal infections led to the emergence and spread of high level tolerance organisms against broad spectrum antibiotics, being crucial to find new antimicrobial agents (Adwan and Mhanna, 2008; Al-Hussaini et al., 2009).

There are some reports on the antitumor potential of *L. nobilis* essential oil (Laizzo et al., 2007; Saab et al., 2013), methanolic (Kaileh et al., 2007), ethanol and aqueous extracts (Al-Kalaldeh et al., 2011), but most publications regard isolated compounds (Panza et al., 2011; Juianti et al., 2012; Lee et al., 2012). Likewise, there is a considering number of reports on the antimicrobial effects, especially on the essential oil of *L. nobilis* (Dadalioğlu et al., 2004; Símic et al., 2004; Santoyo et al., 2006; Curato et al., 2010; Ivanoić et al., 2010; Millezi et al., 2012; Marzouki et al., 2013), but also on its aqueous (Adwan and Mhanna, 2008), ethanolic (Ertuk et al., 2006; Al-Hussaini et al., 2009; Malti and Amarouch, 2009) and methanolic extracts (Fukuyama et al., 2013). The antimicrobial activity of *L. nobilis* isolated molecules is mainly related to terpenes and phenolic compounds (Otsuko et al., 2008; Liu et al., 2009; Fukuyama et al., 2013; Ramos et al., 2013).

Nevertheless, and as far as we know, this is the first study exploring *in vitro* antimicrobial and antitumor activities from cultivated and wild *L. nobilis* enriched phenolic extracts, comparing the differentiated activity of each extract towards specific bacterial and fungal species and also selected human tumor cell lines, using principal component analysis.

3.3.2.2. Materials and methods

Samples

Cultivated *Laurus nobilis* L. samples (leaves) were purchased from Ervital (Castro Daire, Portugal), which produces Mediterranean herbs using organic farming principles and methods. The wild samples (leaves) were collected in Bragança, Portugal, and further lyophilized (FreeZone 4.5, Labconco, Kansas, USA).

Each sample was reduced to a fine dried powder (20 mesh) and stored (7 ºC) until further use.

Standards and reagents

Fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediamine tetraacetic acid), nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, UT, USA). Acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (Saint Louis, USA). Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Dimethylsulfoxide (DMSO), (Merck KGaA, Germany) was used as a solvent. Phosphate buffered saline (PBS) was obtained from Sigma Chemical Co. (St. Louis, USA). Methanol and all other chemicals and

solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Extracts preparation

Methanolic extracts were obtained from cultivated and wild plant material. Each sample (≈1 g) was extracted by stirring with 30 mL of methanol, at room temperature, 150 rpm, for 1 h. The extract was filtered through Whatman no. 4 paper. The residue was then re-extracted with additional 30 mL of methanol. The combined extracts were evaporated at 35 °C (rotary evaporator Büchi R-210, Flawil, Switzerland) to dryness.

For aqueous extracts, plant material (≈1 g) was added to 200 mL of boiling distilled water, left to stand for 5 min out of the heating source and then filtered under reduced pressure. The obtained extract was frozen and lyophilized.

Methanolic and aqueous extracts were redissolved in water (8 mg/mL) or 5% DMSO (10 mg/mL) for antitumor and antimicrobial activity evaluation, respectively. The final solutions were further diluted to different concentrations to be submitted to distinct bioactivity evaluation in *in vitro* assays.

Antitumor activity and hepatotoxicity

Five human tumor cell lines were tested: MCF7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine (MCF7, NCI-H460 and HCT15) or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and HepG2 cells), at 37 ºC, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT15 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates. Sulphorhodamine B assay was performed according to a procedure previously described by the authors (Pereira, Calhelha, Barros and Ferreira, 2013). Ellipticine was used as positive control.

For hepatotoxicity evaluation, a cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, according to an established procedure (Pereira, Calhelha, Barros and Ferreira, 2013); it was designed as PLP2. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and commercial in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Ellipticine was used as positive control. The

results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth).

Antibacterial activity

The following Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973) and Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030) were used. The microorganisms were obtained from the Mycological laboratory, Department of Plant Physiology, Institute for biological research "Sinisa Stanković", University of Belgrade, Serbia.

The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined by the microdilution method. Briefly, fresh overnight culture of bacteria was adjusted by the spectrophotometer to a concentration of 1×10^5 CFU/mL. The requested CFU/mL corresponded to a bacterial suspension determined in a spectrophotometer at 625 nm (OD625). Dilutions of inocula were cultured on solid medium to verify the absence of contamination and check the validity of the inoculum. Different solvent dilutions of methanolic extract/fractions were carried out over the wells containing 100 µL of Tryptic Soy Broth (TSB) and afterwards, 10 µL of inoculum was added to all the wells. The microplates were incubated for 24h at 37 °C. The MIC of each extract was detected following the addition of 40 µL of iodonitrotetrazolium chloride (INT) (0.2 mg/ml) and incubation at 37 °C for 30 min. The lowest concentration that produced a significant inhibition (around 50%) of the growth of the bacteria in comparison with the positive control was identified as the MIC. The minimum inhibitory concentrations (MICs) obtained from the susceptibility testing of various bacteria to tested extract/fraction were determined also by a colorimetric microbial viability assay based on reduction of INT color and compared with positive control for each bacterial strains (CSLI, 2006; Tsukatani et al., 2012). MBC was determined by serial sub-cultivation of 10 µL into microplates containing 100 µL of TSB. The lowest concentration that shows no growth after this sub-culturing was read as the MBC. Standard drugs, namely streptomycin and ampicillin were used as positive controls. DMSO (5%) was used as negative control.

Antifungal activity

For the antifungal bioassays, the following microfungi were used: *Aspergillus fumigatus* (1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), *Penicillium verrucosum* var. *cyclopium* (food isolate) and *Trichoderma viride* (IAM 5061). The organisms were obtained from the Mycological

Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. The micromycetes were maintained on malt agar (MA) and the cultures were stored at +4 °C and subcultured once a month (Booth, 1971).

The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (*v/v*). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 µL/well. The inocula were stored at +4°C for further use. Dilutions of the inocula were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum. Minimum inhibitory concentrations (MICs) determination was performed by a serial dilution technique using 96-well microtitre plates. The extract/fractions were dissolved in 5% solution of DMSO and added to broth malt medium with fungal inoculum. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MIC. The minimum fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 µL in microtitre plates containing 100 µL of malt broth per well and further incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% killing of the original inoculum. Bionazole and ketokonazole were used as positive controls. DMSO (5%) was used as negative control (Espinel-Ingroff, 2001).

Statistical analysis

The extractions were performed in triplicate; each replicate was also measured three times. Data were expressed as means±standard deviations, maintaining the decimal places allowed by the magnitude of standard deviation.

An analysis of variance (ANOVA) with type III sums of squares was performed using the GLM (General Linear Model) procedure of the SPSS software. The dependent variables were analyzed using 2-way ANOVA, with the factors "extract" (E) and "origin" (O). When a statistically significant interaction (E×O) was detected, the two factors were evaluated simultaneously by the estimated marginal means plots for the two levels of each factor. Alternatively, if no statistical significant interaction was verified, means were compared using results obtained for EB and GI were classified using a simple *t*-test (after checking the equality of variances through a Levene's test), since there were fewer than three groups.

Principal components analysis (PCA) was applied as pattern recognition unsupervised classification method. The number of dimensions to keep for data analysis was assessed by the respective eigenvalues (which should be greater than one), by the Cronbach's alpha parameter (that must be positive) and also by the total percentage of variance (that should be as higher as possible) explained by the number of components

selected. The number of plotted dimensions was chosen in order to allow meaningful interpretations.

All statistical tests were performed at a 5% significance level using the SPSS software, version 18.0 (SPSS Inc).

3.3.2.3. Results and Discussion

The interaction effect among *L. nobilis* origin (cultivated or wild) and extract (methanolic or aqueous) was evaluated to understand if both factors act together to cause changes in phenolic composition and/or biological activities. Results are presented as the mean value of each origin (O), comprising both extracts, as well as the mean value of each extract (E) containing sample from both origins. When the interaction among factors (O×E) was significant ($p < 0.05$), acting itself as a source of variability, the comparison of means could not be performed. In these cases, the presented conclusions were drawn from the estimated marginal means (EMM) plots obtained in each case. When the interaction was not significant, a simple *t*-test (fewer than three groups) for equality of means was applied.

Phenolic compound groups present in the studied L. nobilis extracts

Table 30 summarizes the phenolic compound groups present in methanolic and aqueous extracts from cultivated and wild *L. nobilis*, as reported in a previous study of our research group (Dias et al., 2013). The interaction among factors was significant in all cases; nevertheless, some conclusions were obtained from the EMMM plots. In general, cultivated samples had higher contents in total phenolics, especially due to their flavones and flavonols; on the other hand, wild samples had higher contents in flavan-3-ols. All the quantified phenolic compound groups tended to be higher in methanolic extracts, despite the lack of statistical significance for total flavones and total flavonols. Differences among extracts might be due to the higher temperature used in aqueous extracts (Santos-Buelga et al., 2012).

Table 30. Phenolic compounds (mg/g) of different extracts of *Laurus nobilis*. The results are presented as mean±SD.

		Total Flavan 3-ols	Total Flavones	Total Flavonols	Total Phenolic
Origin (O)	Cultivated	56±8	4.4±0.2	26±2	86±11
	Wild	60±4	2.6±0.4	7±2	71±6
	p-value (n=18)	0.025	<0.001	<0.001	<0.001
Extract (E)	Methanolic	63.6±0.4	4±1	19±10	86±11
	Aqueous	52±5	3±1	15±9	70±5
	p-value (n=18)	<0.001	0.104	0.207	<0.001
O×E	*p*-value (n=36)	<0.001	<0.001	<0.001	<0.001

The detailed phenolic profile of all laurel samples was previously described by Dias et al. (2013).

Antitumor activity of the studied L. nobilis extracts

The interaction among factors was again significant in all cases, except MCF7 line (**Table 31**). Considering each factor individually, the origin of laurel had once more higher influence, producing statistically significant differences in all cases except HepG2. Wild bay leaves presented lower GI_{50} values for all cell lines, but also higher toxicity against non-tumor liver primary cells (PLP2; 114 µg/mL); however, GI_{50} concentrations were lower than the hepatotoxic GI_{50} concentration in all cell lines except HepG2, suggesting that this sample could be used for antitumor proposes, at the GI_{50} concentration. Cultivated samples can also be considered for their antitumor activity against NCI-H460, HCT15 and HeLa, since the corresponding GI_{50} values were quite lower than the toxic concentration for PLP2. Differences among aqueous and methanolic extracts were only significant for HCT15 (more susceptible to methanolic extracts), HepG2 (more susceptible to aqueous extracts) and PLP2 primary liver cells (more susceptible to methanolic extracts). Our results for the breast carcinoma cell line (MCF7) showed better results when compared to the essential oil of fruits and leaves of wild *L. nobilis* from Lebanon (>400 µg/mL; Loizzo et al., 2007), but lower activity than aqueous extract from wild bay leaves from Jordan against the same line (88.32% at 50 µg/mL; Al-Kalaldeh et al., 2011). Kaileh et al. (2007) only reported that the methanolic extract of wild bay leaves from Palestine showed no cytotoxicity.

Table 31. Antitumor activity and hepatotoxicity (GI_{50}, µg/mL) of different extracts of *Laurus nobilis*. The results are presented as mean±SD[1].

		MCF7	NCI-H460	HCT15	HeLa	HepG2	PLP2-hepatotoxicity
Origin (O)	Cultivated	187±12 a	83±13	56±1	119±21	185±7	195±85
	Wild	88±5 b	73±19	44±7	69±9	166±59	114±29
	p-value (n=18)	<0.001	0.077	<0.001	<0.001	0.171	<0.001
Extract (E)	Methanolic	140±50	74±21	47±10	100±41	207±17	99±14
	Aqueous	135±53	81±10	53±2	88±11	144±37	210±70
	p-value (n=18)	0.773	0.254	0.011	0.242	<0.001	<0.001
O×E	*p*-value (n=36)	0.261	<0.001	<0.001	<0.001	<0.001	<0.001
Ellipticine		0.91±0.04	1.42±0.01	1.91±0.05	1.14±0.05	3.2±0.5	2.06±0.03

Means within a column with different letters differ significantly (p > 0.001).

Antibacterial activity of the studied L. nobilis extracts

Extract type and origin had a significant interaction in the antibacterial activity against all species except *Micrococcus flavus* (**Table 32**). Cultivated and wild *L. nobilis* were both

active against all bacteria strains with minimal inhibitory concentrations (MIC) of 0.04-0.12 mg/mL and 0.046-0.16 mg/mL, respectively. The minimal bactericidal concentrations (MBC) were higher than MIC, varying from 0.09 to 0.39 mg/mL for cultivated laurel, and from 0.1 to 0.29 mg/mL for wild samples. The effect of laurel origin *per se* was significant for all species except *Staphylococcus aureus* (MIC and MBC), *Escherichia coli* (MBC) and *Enterobacter cloacae* (MBC).

Methanolic extracts were better inhibitors (0.012-0.12 mg/mL) of bacterial growth than the aqueous extracts (0.07-0.20 mg/mL), except for *M. flavus*, whose MIC values did not reveal statistical significance (p=0.858). In all cases, the inhibitory and bactericidal activities were higher than those obtained for the standard ampicillin. In relation to streptomycin, the inhibitory activity of the extracts was also higher, except for *S. aureus* (cultivated, wild and aqueous extracts), *Bacillus cereus* (wild and aqueous extracts) and *Listeria monocytogenes* (aqueous extract). In terms of bactericidal activity, the results were very similar: streptomycin showed higher activity only against *S. aureus* (cultivated, wild and aqueous extracts), *Bacillus cereus* (wild and aqueous extracts) and *L. monocytogenes* (cultivated and aqueous extracts). The bacterial strains more effectively inhibited by cultivated and wild sample were *E. cloacae* and *P. aeruginosa*, respectively; on the other hand, *S. aureus* and *M. flavus* were the most susceptible strains to methanolic and aqueous extracts, respectively. In what regards MBC, the results were the same except for aqueous extract, which proved to have the highest bactericidal effect against *E. cloacae*.

Table 32. Antibacterial activity (MIC and MBC, mg/mL) of different extracts of *Laurus nobilis*. The results are presented as mean±SD[1].

		Staphylococcus aureus	*Bacillus cereus*	*Micrococcus flavus*	*Listeria monocytogenes*	*Pseudomonas aeruginosa*	*Salmonella typhimurium*	*Escherichia coli*	*Enterobacter cloacae*
					MIC				
Origin (O)	Cultivated	0.06±0.04	0.08±0.04	0.048±0.005 b	0.1±0.1	0.08±0.04	0.08±0.03	0.12±0.02	0.04±0.01
	Wild	0.05±0.05	0.11±0.01	0.101±0.005 a	0.16±0.05	0.046±0.003	0.11±0.01	0.16±0.05	0.08±0.05
	p-value (n=18)	0.619	0.001	<0.001	0.030	0.001	<0.001	0.002	0.004
Extract (E)	Methanolic	0.012±0.005	0.08±0.04	0.08±0.03	0.06±0.04	0.046±0.003	0.07±0.03	0.12±0.02	0.03±0.01
	Aqueous	0.10±0.01	0.11±0.01	0.07±0.03	0.20±0.02	0.08±0.03	0.11±0.01	0.16±0.05	0.08±0.05
	p-value (n=18)	<0.001	0.001	0.858	<0.001	0.001	<0.001	0.009	0.002
O×E	*p*-value (n=36)	0.002	<0.001	0.212	<0.001	<0.001	<0.001	<0.001	<0.001
Ampicillin		0.25±0.02	0.25±0.03	0.25±0.04	0.37±0.05	0.74±0.05	0.37±0.02	0.25±0.01	0.37±0.04
Streptomycin		0.04±0.01	0.09±0.01	0.17±0.02	0.17±0.01	0.17±0.01	0.17±0.02	0.17±0.02	0.26±0.03
					MBC				
Origin (O)	Cultivated	0.16±0.04	0.15±0.04	0.11±0.01 b	0.39±0.02	0.18±0.02	0.16±0.05	0.20±0.02	0.09±0.03
	Wild	0.1±0.1	0.19±0.02	0.20±0.01 a	0.29±0.05	0.10±0.01	0.21±0.02	0.2±0.1	0.1±0.1
	p-value (n=18)	0.262	0.001	<0.001	<0.001	<0.001	0.002	0.764	0.111
Extract (E)	Methanolic	0.07±0.05	0.15±0.04	0.16±0.05	0.3±0.1	0.14±0.04	0.17±0.05	0.1±0.1	0.08±0.04
	Aqueous	0.21±0.02	0.19±0.01	0.15±0.05	0.37±0.03	0.15±0.05	0.21±0.01	0.3±0.1	0.1±0.1
	p-value (n=18)	<0.001	<0.001	0.461	0.005	0.696	0.008	<0.001	0.041
O×E	*p*-value (n=36)	<0.001	<0.001	0.719	<0.001	<0.001	<0.001	<0.001	<0.001
Ampicillin		0.37±0.04	0.37±0.05	0.37±0.04	0.49±0.05	1.2±0.1	0.49±0.05	0.49±0.04	0.74±0.05
Streptomycin		0.09±0.01	0.17±0.02	0.34±0.05	0.34±0.04	0.34±0.03	0.34±0.05	0.34±0.04	0.52±0.05

[1]Means within a column with different letters differ significantly (p > 0.05).

All presented MIC results were much better that those obtained by Al-Hussaini et al. (2009) on the ethanolic extracts of *L. nobilis* from Jordan against *S. aureus*, *B. cereus*, *E. coli*, *S. typhimurium* and *P. aeruginosa*. The same applies to the results obtained by Malti & Amarouch (2009) on the ethanolic extracts of leaves of bay laurel from Morocco against *B. cereus*, *S. aerus*, *L. monocytogenes*, *E. cloacae*, *E. coli* and *P. aeruginosa* (> 2 mg/mL). And further to the results obtained on the essential oils of bay leaves from Turkey against *E. coli*, *S. aureus*, and *P. aeruginosa* that showed MIC values of 5 mg/mL (Dadalioğlu et al., 2004). Adwan & Mhanna (2008) obtained better results with aqueous extracts of bay leaves form Palestine against *S. aureus* bacterial strain ($<6.1\times10^{-3}$ mg/L), but only when conjugated with enrofloxacin and cephalexin antibiotics.

Antifungal activity of the studied L. nobilis extracts

The interaction among factors was once more significant in almost all cases, excepting MIC values for *Penicillium ochrochloron* (p=0.278) and MBC values for *Aspergillus niger* (p=0.312) and *P. ochrochloron* (p=0.052) (**Table 33**). All samples showed activity against all fungal strains. The inhibitory activity on fungal growth was more affected by extract type, as it can be concluded from the statistically significant differences verified in all cases, except *A. ochraceus* (p=0.077). There was not a better extract for all cases: methanolic extracts were more active against *A. versicolor*, *Trichoderma viride*, *P. funiculosum* and *P. ochrochloron*, while aqueous extracts were better in all remaining cases (except, of course, *A. ochraceus*, which gave no differences). Cultivated and wild samples gave MIC varying from 0.01 to 0.17 mg/mL and from 0.02 to 0.3 mg/mL, respectively. In the cases revealing statistically significant differences, cultivated laurel samples gave higher inhibitory activity.

In what concerns fungicidal activity, MFC varied among 0.03 and 0.6 mg/mL for cultivated laurel and 0.03-0.5 mg/mL for wild samples. *A. versicolor*, *A. niger* and *T. viride* were equally inhibited by cultivated and wild laurel. Comparing extract types, MFC varied from 0.016 to 0.7 mg/mL, for methanolic extract and 0.046 to 0.3 mg/mL, for aqueous extracts. Like it was observed for inhibitory activity, the fungicidal action was more affected by the type of extract when compared with laurel origin (except *P. funiculosum*).

Table 33. Antifungal activity (MIC and MFC, mg/mL) of different extracts of *Laurus nobilis*. The results are presented as mean±SD[1].

		Aspergillus fumigatus	*Aspergillus versicolor*	*Aspergillus ochraceus*	*Aspergillus niger*	*Trichoderma viride*	*Penicillium funiculosum*	*Penicillium ochrochloron*	*Penicillium verrucosum*
					MIC				
Origin (O)	Cultivated	0.07±0.05	0.01±0.01	0.04±0.01	0.3±0.2	0.02±0.01	0.03±0.01	0.12±0.02	0.17±0.05
	Wild	0.2±0.1	0.02±0.01	0.048±0.004	0.3±0.2	0.02±0.01	0.03±0.02	0.11±0.02	0.20±0.02
	p-value (n=18)	<0.001	0.005	<0.001	0.603	0.163	0.407	0.054	0.005
Extract (E)	Methanolic	0.2±0.1	0.009±0.003	0.04±0.01	0.47±0.01	0.008±0.005	0.017±0.005	0.10±0.01 b	0.20±0.02
	Aqueous	0.06±0.04	0.024±0.005	0.045±0.002	0.07±0.04	0.029±0.002	0.048±0.002	0.12±0.02 a	0.17±0.05
	p-value (n=18)	<0.001	<0.001	0.077	<0.001	<0.001	<0.001	0.008	0.007
O×E	*p*-value (n=36)	<0.001	0.003	<0.001	<0.001	<0.001	<0.001	0.278	<0.001
Bifonazole		0.15±0.01	0.10±0.01	0.15±0.02	0.15±0.01	0.15±0.01	0.20±0.03	0.20±0.02	0.10±0.01
Ketoconazole		0.20±0.02	0.20±0.03	1.5±0.1	0.20±0.02	1.0±0.1	0.20±0.02	2.5±0.3	0.20±0.04
					MFC				
Origin (O)	Cultivated	0.2±0.1	0.05±0.03	0.08±0.03	0.4±0.4	0.03±0.01	0.10±0.02	0.23±0.02 a	0.6±0.3
	Wild	0.4±0.1	0.04±0.01	0.11±0.01	0.5±0.3	0.03±0.02	0.11±0.02	0.20±0.02 b	0.40±0.03
	p-value (n=18)	<0.001	0.091	<0.001	0.196	0.500	0.027	<0.001	0.041
Extract (E)	Methanolic	0.3±0.1	0.021±0.004	0.08±0.03	0.7±0.3 a	0.016±0.004	0.10±0.02	0.20±0.03 b	0.6±0.2
	Aqueous	0.2±0.1	0.06±0.02	0.11±0.01	0.2±0.1 b	0.046±0.002	0.11±0.01	0.23±0.02 a	0.3±0.1
	p-value (n=18)	<0.001	<0.001	<0.001	<0.001	<0.001	0.122	<0.001	<0.001
O×E	*p*-value (n=36)	0.001	<0.001	<0.001	0.312	<0.001	<0.001	0.052	<0.001
Bifonazole		0.20±0.02	0.20±0.03	0.20±0.01	0.20±0.02	0.20±0.04	0.25±0.05	0.25±0.04	0.20±0.03
Ketoconazole		0.50±0.05	0.50±0.04	2.0±0.4	0.50±0.05	1.0±0.1	0.50±0.04	3.5±0.5	0.30±0.05

[1]Means within a column with different letters differ significantly (p > 0.05).

For both samples and both extracts, *A. fumigatus* (only cultivated and aqueous samples in the case of bifonazole), *A. versicolor*, *A. ochraceus*, *T. viride*, *P. funiculosum* and *P. ochrochloron* showed better activity than bifonazole and ketoconazole. *A. versicolor* and *T. viride* were the most susceptible fungal strains, while *A. niger* and *P. verrucosum* were the most resistant. Al-Hussaini et al. (2008) and Simić et al. (2004) showed better results on ethanolic extracts and essential oil, respectively, of laurel leaves from Jordan and Serbia and Montenegro against *A. niger*.

Principal component analysis (PCA)

After analysing individually each bioactivity indicator and phenolic compound contents, PCA was applied to obtain an overview of main differences verified among cultivated and wild *L. nobilis* samples, as well as among the methanolic and aqueous extracts. The plot of component loadings for extract type was designed with the first two dimensions (first: Cronbach's α, 0.965; eigenvalue, 17.194; second: Cronbach's α, 0.950; eigenvalue, 13.721), which included most variance of data (first: 40.94%; second: 32.67%); third and fourth dimensions were also significant, but their plotting would give a complex output. Objects distribution (**Figure 17**) indicates a clear separation of methanolic from aqueous extracts. Furthermore, objects corresponding to wild and cultivated samples were clearly separated within each type of extract. The assignment of each set of objects to either wild or cultivated samples was done according to the tabled object scores (data not shown).

Group corresponding to cultivated samples extracted with methanol (solid grey line ellipse) was characterized by the high amounts bioactive compounds, specifically flavonols, flavones and total phenolics, and its high bioactivity against *B. cereus* (MIC and MBC), *Salmonella typhimurium* (MIC and MBC), *E. coli* (MIC), *E. cloacae* (MIC), *L. monocytogenes* (MIC), *A. ochraceus* (MIC and MFC), *A.* (MIC) and *P. funicolusum* (MFC).

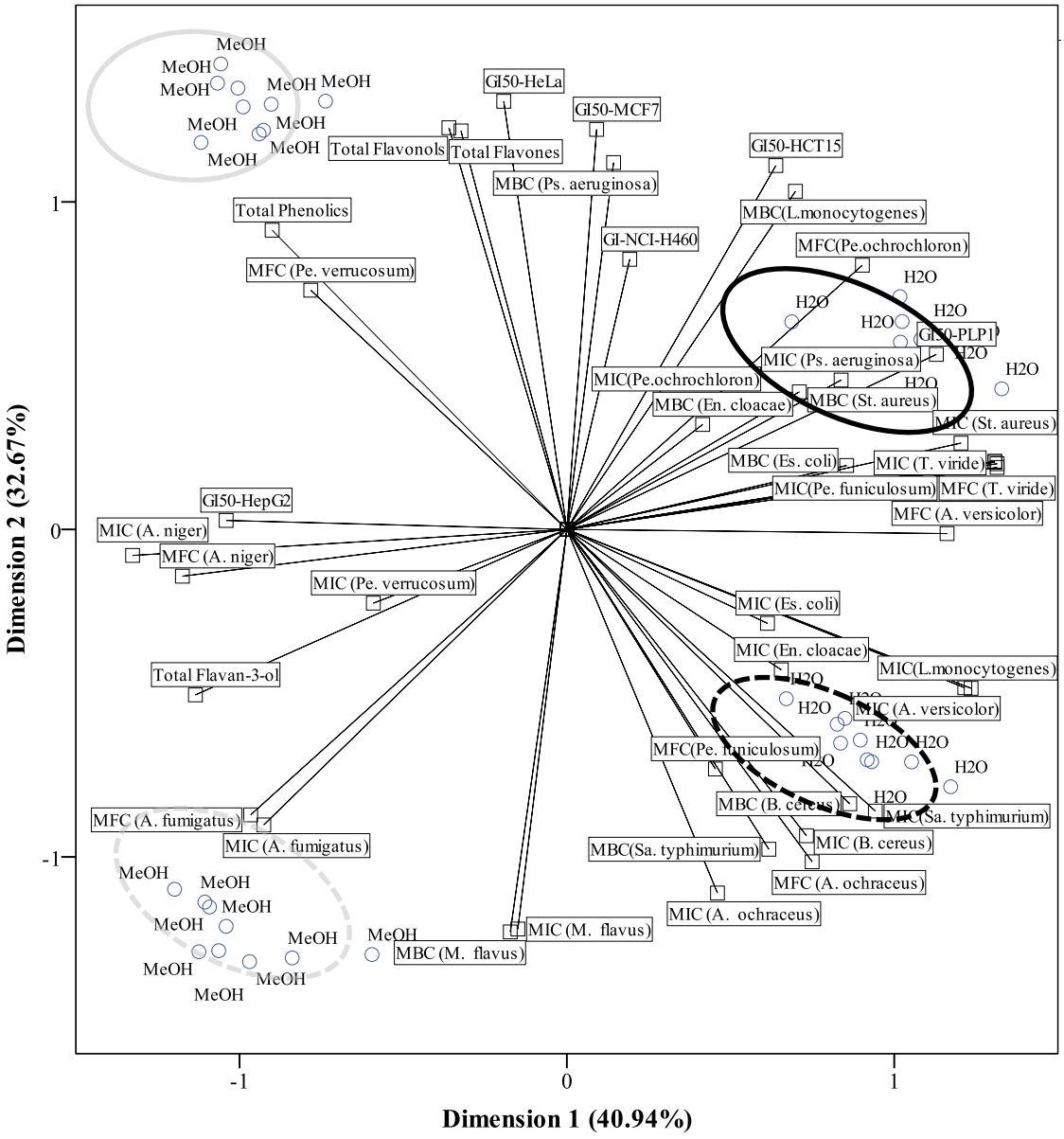

Figure 17. Biplot of objects (extraction solvents) and component loadings (evaluated parameters).

The most distinctive features in cultivated samples extracted with water (solid black line ellipse) were the low content in flavan-3-ols, the low inhibitory activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Penicillium funicolusum*, P. ochrochloron and *Tricholoma viride*, low bactericidal activity towards *E. coli* (MIC), *E. cloacae*, *L. monocytogenes* and *S. aureus,* low fungicidal activity against (*A. versicolor*, *P. ochrochloron* and *Tricholoma viride*) and low toxicity against HCT15 and PLP2. This extract was particularly active towards HepG2, *A. fumigatus* and *A. flavus*.

A third group (dashed grey line ellipse), corresponding to wild samples extracted with methanol, was characterized as having an activity opposite to that demonstrated by cultivated samples extracted with water; *i.e.*, it has the worst activity against *A. fumigatus* and

A. flavus, but showed to be quite active on the bacteria, fungi and tumor cell lines less susceptible to the aqueous extracts from cultivated samples, containing also the higher quantities of flavan-3-ols. The content in flavan-3-ols might be related to their high bioactivity, especially against bacteria. It could also indicate that the fungi *A. fumigatus* and *A. flavus* are poorly susceptible to flavan-3-ols.

Similarly, wild samples extracted with water (dashed black line ellipse) had the reverse behavior in comparison to cultivated samples extracted with methanol. This particular group was mostly active against *P. verrucosum,* but it showed the worst activity against *B. cereus* (MIC and MBC), *Salmonella typhimurium* (MIC and MBC), *E. coli* (MIC), *E. cloacae* (MIC), *L. monocytogenes* (MIC), *A. ochraceus* (MIC and MFC), *A.* (MIC) and *P. funicolusum* (MFC) and also the lowest contents in flavonols, flavones and total phenolics.

3.3.2.4. Conclusions

The extract type induced the most marked changes in bioactivity of laurel samples. Furthermore, each of the assayed factors (origin and extract type) act in a differentiated manner; *i.e.*, the same evaluated parameter gave sometimes statistically significant differences regarding laurel origin, but no effect at all from extract type, or *vice versa*. From the PCA biplot, it became clear that wild bay leaves samples were more effective to inhibit tumor cell lines growth, especially HeLa, MCF7, NCI-H460 and HCT15. HepG2, as previously highlighted, had the same response to bay leaves from wild and cultivated origin. It was also observed that methanolic extracts tended to have higher antimicrobial activity, except *A. niger*, *A. fumigatus* and *P. verrucosum.* The differences in bioactivity might be related to the higher phenolic compounds contents presented by methanolic extracts.

The most interesting finding in this work was the bioactive specificity of each laurel extract, considering its wild or cultivated origin. In fact, from the obtained results it is possible to choose the combination extract type/origin with potentially highest effect against determined bacteria, fungi or tumor cell line.

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3.3.2.5. References

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3.4. *Taraxacum* sect. Ruderalia

Neste sub-capítulo apresenta-se a caraterização nutricional e química, e as propriedades antioxidantes e citotóxicas de *Taraxacum* sect. Ruderalia silvestre e das respetivas infusões, decocções e extratos metanol: água.

3.4.1. Composição nutricional, atividade antioxidante e compostos fenólicos de *Taraxacum* sect. Ruderalia silvestre

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Nutritional composition, antioxidant activity and phenolic compounds of wild *Taraxacum* sect. *Ruderalia*

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Abstract

Flowers and vegetative parts of wild *Taraxacum* identified as belonging to sect. *Ruderalia* were chemically characterized in nutritional composition, sugars, organic acids, fatty acids and tocopherols. Furthermore, the antioxidant potential and phenolic profiles were evaluated in the methanolic extracts, infusions and decoctions. The flowers gave higher content of sugars, tocopherols and flavonoids (mainly luteolin *O*-hexoside and luteolin), while the vegetative parts showed higher content of proteins and ash, organic acids, polyunsaturated fatty acids (PUFA) and phenolic acids (caffeic acid derivatives and especially chicoric acid). In general, vegetative parts gave also higher antioxidant activity, which could be related to the higher content in phenolic acids (R^2=0.9964, 0.8444, 0.4969 and 0.5542 for 2,2-diphenyl-1-picrylhydrazyl, reducing power, β-carotene bleaching inhibition and thiobarbituric acid reactive substances assays, respectively). Data obtained demonstrated that wild plants like *Taraxacum*, although not being a common nutritional

reference, can be used in an alimentary base as a source of bioactive compounds, namely antioxidants.

Keywords: T*araxacum* sect. *Ruderalia*; Wild; Nutritional Value; Antioxidants contribution

3.4.1.1. Introduction

Wild medicinal plants are used by the majority of the world's population and, therefore, still represent a milestone for ethnomedicine in the search for new and safer bioactive compounds. Beyond their nutritional properties, medicinal plants provide beneficial health effects due to the presence of antioxidant compounds and other nutraceuticals (Fabricant & Farnsworth, 2001; Bernal, Mendiola, Ibáñez & Cifuentes, 2011).

The vast genus of *Taraxacum*, commonly known as dandelion, is divided in several sections, each one with many species of this plant; Ruderalia is the largest and most widespread section (Meirmans, Calama, Bretagnolle, Felber, & Nijs, 1999). This plant genus, commonly found in the warm temperate zone of the northern hemisphere (Schütz, Carle & Schieber, 2006), is used since ancient times in folk medicine to treat dyspepsia, spleen and liver complaints, breast and uterus diseases, anorexia, but also in lactating, diuretic, and anti-inflammatory remedies (Schütz et al., 2006; Jeon et al., 2008). The young leaves and flowers are very appreciated in salads, while roasted roots are used as substitutes of coffee. They are also consumed as infusion and decoction to treat some illness (Schütz et al., 2006; Sweeney, Vora, Ulbricht & Basch, 2005; Mlcek & Rop, 2011).

The majority of reports found in literature is focused in a particular species, *T. officinalis*, and describe antioxidant properties (Hu & Kitts, 2003 and 2005; Hudec et al., 2007; Jeon et al., 2008), nutritional value (Escudero, Arellano, Fernández, Albarracín, & Mucciarelli, 2003) and fatty acids (Liu, Howe, Zhou, Hocart, & Zhang, 2002). The same occurs regarding phenolic profile being flavonoid glycosides and hidroxycinammic acids, mainly chicoric acid, reported as the most abundant compounds (Williams, Goldstone, & Greenham, 1996; Gatto et al., 2011). *T. obovatum* and *T. mongolicum* were characterized in terms of organic acids (Sánchez-Mata et al., 2012) and phenolic compounds (Shi et al., 2007; Shi, Zhang, Zhao, & Huang, 2008), respectively.

Nevertheless, there is a lack of information regarding chemical and bioactive properties of many species of *Taraxacum* genus. Considering the medicinal properties reported for the genus, the combination of functional and nutritional characteristics should be explored (Guarrera & Savo, 2013). In this perspective, flowers and vegetative parts of wild *Taraxacum*, identified as belonging to section Ruderalia (endemic from Iberian Peninsula), were chemically characterized regarding nutritional value, free sugars, organic acids, fatty

acids and tocopherols. Furthermore, the antioxidant activity of its methanolic extract, infusion and decoction was correlated to the individual phenolic profile, in order to highlight the duality of medicinal plants in terms of nutritional composition and bioactive features.

3.4.1.2. Materials and methods

Samples

Flowers and vegetative parts of wild *Taraxacum* sect. Ruderalia (Supplementary Material) were collected in Bragança, North-eastern Portugal, in April 2012. Key morphological characters from Flora Iberica (http://www.rjb.csic.es/floraiberica/) were used for plant identification. Voucher specimens (nº 9686) are available in Escola Superior Agrária de Bragança Herbarium (BRESA). The samples were further lyophilized (FreeZone 4.5, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain homogenate samples.

Nutritional contribution

Proximate composition and energetic value. The samples were analyzed for proteins, fat, carbohydrates and ash using the AOAC procedures (AOAC, 1995). Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein) + 3.75 × (g carbohydrate) + 9 × (g fat).

Sugars. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) (Pereira, Barros, Carvalho & Ferreira, 2011) using melezitose as internal standard (IS). The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method.

Organic acids. Organic acids were determined by high performance liquid chromatography coupled to a PDA detector using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths (Pereira, Barros, Carvalho, & Ferreira, 2013). For quantitative analysis, calibration curves were prepared from oxalic, quinic malic, ascorbic, citric and fumaric acid standards.

Fatty acids. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID)/capillary column (Dias, Barros, Sousa, & Ferreira, 2013). Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards.

Tocopherols. Tocopherols were determined by HPLC coupled to a fluorescence detector (Pereira et al., 2011). The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal

response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards.

Antioxidants contribution

Methanolic extracts, infusions and decoctions preparation.

All the preparations were obtained either from lyophilized powder of flowers or vegetative parts. Each sample (1 g) was extracted twice by stirring with 30 mL of methanol (25 ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The combined methanolic extracts were evaporated at 40 ºC (rotary evaporator Büchi R-210) to dryness.

For infusion preparation the sample (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure. For decoction preparation the sample (1 g) was added to 200 mL of distilled water, heated (heating plate, VELP scientific) and boiled for 5 min. The mixture was left to stand for 5 min and then filtered under reduced pressure. The obtained infusions and decoctions were frozen and lyophilized.

Methanolic extracts and lyophilized infusions and decoctions were redissolved in methanol and water, respectively (final concentration 5 mg/mL) for antioxidant activity evaluation. For toxicity assay, the extracts were redissolved in water at 8 mg/mL. The final solutions were further diluted to different concentrations to be submitted to the antioxidant and toxicity assays.

Antioxidant activity evaluation.

The antioxidant activity was evaluated by DPPH radical-scavenging activity, reducing power, inhibition of β-carotene bleaching in the presence of linoleic acid radicals and inhibition of lipid peroxidation using TBARS in brain homogenates (Dias et al., 2012). Trolox was used as positive control.

Phenolic profile.

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) (Rodrigues et al., 2012). Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared from caffeic acid, luteolin-7-*O*-glucoside and quercetin-3-*O*-glucoside standards.

Evaluation of toxicity in a primary culture of porcine liver cells

A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, according to an established procedure (Abreu et al., 2011); it was designed as PLP2. The cell growth was followed by using Sulphorhodamine B assay.

Statistical analysis

For each part (flowers or vegetative parts), three samples were used and all the assays were carried out in triplicate. The results were expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 18.0 program.

3.4.1.3. Results and Discussion

Nutritional contribution

The results obtained for macronutrients, sugars, organic acids, fatty acids and tocopherols of flowers and vegetative parts of *Taraxacum* sect. *Ruderalia* are presented in **Table 34**. Carbohydrates (including fiber) were the major macronutrients found in both samples (similar amounts). Vegetative parts showed higher levels of proteins and ash, while flowers gave higher fat content and energy value. Escudero et al. (2003) studied the nutritional value of flour of *T. officinale* leaves from Argentina, and also reported high levels of carbohydrates and proteins (58.35 g/100 g dw and 15.48 g/100 g dw, respectively).

Fructose, glucose and sucrose were found in both flowers and vegetative parts, although flowers presented higher levels of fructose, sucrose and total sugars; trehalose and raffinose were not detected in this sample.

The highest level of total organic acids was found in vegetative parts, being oxalic acid the major one followed by malic acid; ascorbic acid was also found but in very low amounts (probably related to some degradation between the field collection and the lyophilisation of the fresh samples); quinic acid was not found in vegetative parts. Sánchez-Mata et al. (2012), studied the composition in organic acids of the basal leaves of wild *T. obovatum*, reporting the same compounds, but with malic acid as the major organic acid found, followed by ascorbic acid.

Up to twenty-six fatty acids were found in *Taraxacum* flowers, with linoleic acid (C18:2n6c) as the majority fatty acid followed by α-linolenic acid (C18:3n3). The vegetative parts showed only twenty fatty acids, being α-linolenic acid (C18:3n3) the main fatty acid followed by linoleic acid (C18:2n6c), the opposite of the observed in flowers sample. Liu et al. (2002) obtained similar results for young leaves of *T. officinale* from Australia, being α-

linolenic acid the predominant one (223 mg/100 g fw). The flour of *T. officinale* leaves also showed α-linolenic acid (34.61%) as the major fatty acid (Escudero et al., 2003). In our study, both flowers and vegetative parts presented higher contents of polyunsaturated fatty acids (PUFA) than saturated fatty acids (SFA), which increases their phytochemical value, as some PUFA are essential nutrients and have been involved in the prevention of important chronic diseases (Alonso & Maroto, 2000).

The flowers of dandelion presented higher levels of individual (mainly α- tocopherol) and total tocopherols than vegetative parts, in which δ-tocopherol was not found.

Table 34. Macronutrients, free sugars, organic acids, fatty acids and tocopherols of flowers and vegetative parts of *Taraxacum* sect. *Ruderalia*.

	Flowers	**Vegetative parts**
Moisture (g/100 g fw)	77.43 ± 2.07[b]	79.12 ± 2.04[a]
Fat (g/100 g dw)	6.56 ± 0.15[a]	2.96 ± 0.00[b]
Proteins (g/100 g dw)	15.13 ± 1.22[b]	18.26 ± 0.90[a]
Ash (g/100 g dw)	0.86 ± 0.02[b]	1.44 ± 0.04[a]
Carbohydrates (g/100 g dw)	77.46 ± 1.28[a]	77.35 ± 0.89[a]
Energy (kcal/100 g dw)	429.36 ± 0.47[a]	409.07 ± 0.10[b]
Fructose	4.71 ± 0.32[a]	0.29 ± 0.02[b]
Glucose	1.81 ± 0.10[b]	2.08 ± 0.19[a]
Sucrose	6.88 ± 0.20[a]	3.65 ± 0.25[b]
Trehalose	Nd	0.31 ± 0.05
Raffinose	Nd	0.19 ± 0.03
Total sugars (g/100 g dw)	13.4 ± 0.62[a]	6.53 ± 0.47[b]
Oxalic acid	0.96 ± 0.01[b]	4.76 ± 0.04[a]
Quinic acid	0.07 ± 0.01	nd
Malic acid	2.12 ± 0.06[b]	4.58 ± 0.14[a]
Ascorbic acid	0.07 ± 0.00[b]	0.04 ± 0.00[a]
Citric acid	1.34 ± 0.03[a]	0.66 ± 0.00[b]
Fumaric acid	0.02 ± 0.00[a]	0.02 ± 0.00[a]
Total organic acids (g/100 g dw)	4.55 ± 0.10[b]	10.05 ± 0.10[a]
Fatty acid		
C16:0	17.01 ± 3.12	10.09 ± 2.06
C18:2n6c	33.03 ± 1.33	24.21 ± 1.86
C18:3n3	23.14 ± 1.17	57.38 ± 4.96
SFA	33.53 ± 4.12[a]	14.99 ± 2.73[b]
MUFA	2.97 ± 0.00[a]	2.20 ± 0.04[b]
PUFA	63.50 ± 4.11[b]	82.82 ± 2.77[a]
PUFA/MUFA	1.92 ± 0.36[b]	5.64 ± 1.21[a]
n6/n3	1.12 ± 0.06[a]	0.44 ± 0.08[b]
α – tocopherol	21.60 ± 1.76[a]	16.85 ± 1.26[b]
β – tocopherol	11.24 ± 0.93[a]	0.64 ± 0.12[b]
γ – tocopherol	5.61 ± 0.54[a]	1.70 ± 0.23[b]
δ – tocopherol	6.31 ± 0.78	nd
Total tocopherols (g/100 g dw)	44.76 ± 4.02[a]	19.19 ± 1.61[b]

nd- not detected; fw- fresh weight; dw- dry weight. In each row different letters mean significant differences (p□0.05). Palmitic acid (C16:0); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3); SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

Antioxidants contribution

The antioxidant activity of methanolic extracts, infusions and decoctions of flowers and vegetative parts of *Taraxacum* sect. *Ruderalia* was studied and the results are presented in **Table 35**. The decoction of vegetative parts showed the highest DPPH scavenging activity and reducing power. The decoction of flowers, and the infusion and decoction of vegetative parts showed statistically similar results for β-carotene bleaching inhibition. The methanolic extract and infusion of vegetative parts showed the highest activity in TBARS (thiobarbituric acid reactive substances) assay presenting EC_{50} values without significant differences. Hu & Kitts (2005 and 2003) and Hudec et al. (2007), reported higher DPPH scavenging activity of different extracts from *T. officinale*. Otherwise, Jeon et al. (2008) reported a lower activity for ethanolic extracts of aerial parts of *T. officinale* from Korea. Nevertheless, these results are very difficult to compare with the herein described, due to the differences in the extraction solvents and methodologies. Furthermore, it should be highlighted that, up to 400 µg/mL, the extracts did not show toxicity for a liver cells primary culture (**Table 35**).

Table 35. Antioxidant activity of methanolic extracts, infusions and decoction of flowers and vegetative parts of *Taraxacum* sect. *Ruderalia*.

	Flowers			Vegetative parts		
	Methanolic	**Infusion**	**Decoction**	**Methanolic**	**Infusion**	**Decoction**
Extraction yield (%)	29.8 ± 3.10	21.8 ± 0.15	23.4 ± 3.23	27.6 ± 2.70	20.15 ± 2.85	21.60 ± 1.52
DPPH scavenging activity (EC_{50}, mg/mL)	0.80 ± 0.01^{b}	0.53 ± 0.12^{c}	0.42 ± 0.03^{d}	0.89 ± 0.03^{a}	0.35 ± 0.03^{d}	0.12 ± 0.00^{e}
Reducing power (EC_{50}, mg/mL)	0.41 ± 0.01^{b}	0.30 ± 0.00^{d}	0.47 ± 0.01^{a}	0.39 ± 0.01^{c}	0.31 ± 0.02^{d}	0.16 ± 0.00^{e}
β-carotene bleaching inhibition (EC_{50}, mg/mL)	1.89 ± 0.09^{b}	2.63 ± 0.70^{a}	0.40 ± 0.09^{c}	1.61 ± 0.58^{b}	0.46 ± 0.03^{c}	0.76 ± 0.09^{c}
TBARS inhibition (EC_{50}, mg/mL)	0.39 ± 0.08^{c}	0.23 ± 0.02^{d}	0.60 ± 0.02^{b}	0.13 ± 0.02^{e}	0.16 ± 0.03^{e}	0.71 ± 0.08^{a}
PLP2- liver cells primary culture (GI_{50}, µg/mL)	> 400	> 400	> 400	> 400	> 400	> 400

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI50 > 400 indicates that no toxicity was found when testing samples up to 400 µg/mL. In each row different letters mean significant differences ($p<0.05$).

The main phenolic compounds found in the flowers and vegetative parts of *Taraxacum* sect. *Ruderalia* methanolic extracts, infusions and decoctions were phenolic acids and derivatives, as also flavonoids such as flavonols and flavones (**Table 36**).

Trans-caffeic acid (peak 4 in flowers and 6 in vegetative parts), and 5-*O*-caffeoylquinic acid (compound 3 in both parts) were positively identified by comparison of their MS fragmentation patterns, UV spectra and retention times with commercial standards.

Compound 7 in vegetative parts was assigned to *cis*-caffeic acid, based on its UV and mass spectral characteristics and elution order when compared to compound 6.

Compounds 1 ($[M-H]^-$ at *m/z* 311) and 2 ($[M-H]^-$ at *m/z* 341) in both samples were assigned as caffeic acid pentoside and hexoside, respectively. This identification was based on their product ion at *m/z* 179 ($[caffeic\ acid-H]^-$) resulting from the loss of 132 u and 162 u (pentosyl and hexosyl residue, respectively), and it is also supported by their UV spectra characteristic of caffeic acid derivatives. Peaks 10 and 11 in flowers and 16 in vegetative parts ($[M-H]^-$ at *m/z* 515) corresponded to dicaffeoylquinic acids and were identified based on their elution order and MS^2 fragmentation patterns as described by Clifford, Johnston, Knight, & Kuhnert (2003 and 2005). Thus, peak 10 in flowers and 16 in vegetative parts were identified as 3,5-*O*-dicaffeoylquinic acid, producing an MS^2 base peak at *m/z* 353 from the loss of one of the caffeoyl moieties $[M-H-caffeoyl]^-$, whose subsequent fragmentation yielded product ions characteristic of monocaffeoylquinic acids at *m/z* 191, 179, 173 and 135, although in the case of the dicaffeoyl derivative with a comparatively more intense signal at *m/z* 179 (56%-63% of base peak). Peak 11 in flowers was assigned to 4,5-*O*-dicaffeoylquinic acid according to its elution order and MS^2 fragmentation, with an MS^2 base peak at *m/z* 353 ($[M-H-caffeoyl]^-$) and another intense signal at *m/z* 173, from the loss of a second caffeoyl moiety, characteristic of isomers substituted at position 4 (Clifford et al., 2003, 2005).

Compounds 5 and 6 in flowers and 10 and 11 in vegetative parts showed the same pseudomolecular ion ($[M–H]^-$ at *m/z* 473) and a fragmentation pattern that allowed assigning them as chicoric acid (dicaffeoyltartaric acid) isomers. Two chicoric acid isomers were also reported by Schütz, Kammerer, Carle, & Schieber (2005) in dandelion (*Taraxacum officinale* WEBER ex F.H.WIGG.) showing similar fragmentation behavior although with different abundances of the released product ions. In the case of Schütz and coworkers the ion was at *m/z* 311 (loss of a caffeoyl moiety) appeared as MS^2 base peak (100% abundance), whereas in our study major fragments were observed at *m/z* 179 ($[caffeic\ acid-H]^-$) and 149 ($[tartaric\ acid-H]^-$). Furthermore, in vegetative parts, peak 4, showing a pseudomolecular ion at *m/z* 635, 162 u greater than chicoric acids and with similar product ions, was identified as a chicoric acid hexoside.

Compounds 7, 8, 9, 12-14 in flowers and 12 and 14 in vegetative parts were identified as luteolin derivatives. Peaks 8 (flowers) and 14 (vegetative parts) were positively identified as luteolin 7-*O*-glucoside, and compound 13 (flowers) was identified as luteolin, by comparison of their MS and UV spectra and retention characteristics with commercial standards. The rest of luteolin derivatives were tentatively identified as luteolin *O*-rutinoside (peaks 7 in flowers and 12 in vegetative parts), luteolin *O*-hexoside (peak 9 in flowers) and luteolin *O*-acetylhexoside (peak 12 in flowers), based on their pseudomolecular ions and MS^2

fragment losses corresponding to rutinosyl (-308 u), hexosyl (-162 u) and acetylhexosyl (-42-162 u) moieties, respectively.

The remaining phenolic compounds in vegetative parts that can be attributed to quercetin derivatives (λ_{max} around 350 nm and an MS^2 fragment at *m/z* 301). Compounds 5 and 8 ($[M-H]^-$ at *m/z* 595) were identified as quercetin containing a pentosyl and a hexosyl residues. The observation of only a MS^2 fragment at *m/z* 463 from the loss of a pentosyl moiety (-132 u) suggests that both sugars were constituting a disaccharide that would be linked to the aglycone through the hexose, otherwise a fragment from the loss of a hexosyl residue (-162 mu) should have been observed. These peaks were tentatively identified as quercetin *O*-pentosyl hexosides bearing the sugar moiety located at different position on the aglycone. Peak 15 ($[M-H]^-$ at *m/z* 505) corresponded to a quercetin *O*-acetylhexoside according to its pseudomolecular ion and MS^2 fragment released at *m/z* 301 (quercetin; $[M-H-42-162]^-$, loss of an acetylhexoside moiety). Peak 9 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 667, 162 u greater than peak 15 indicating the presence of an additional hexosyl moiety. The formation of fragments due to the alternative loss of a hexosyl moiety (*m/z* at 505) and an acetylhexosyl moiety (*m/z* at 463) suggested that both residues were located at different positions on the aglycone, so that it was assigned to quercetin *O*-hexoside-*O*-acetylhexoside. Finally, peak 13, with an $[M-H]^-$ at *m/z* 433, releasing only a product ion at *m/z* 301 (quercetin; [M-H-132]-, loss of a pentosyl moiety) was assigned to s a quercetin *O*-pentoside.

Table 36. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification of flavonoids and phenolic acids in flowers and vegetative parts of wild *Taraxacum* sect. *Ruderalia*.

Flowers

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g extract)		
						Methanolic	Infusion	Decoction
1	5.5	330	311	179(100), 135(94)	Caffeic acid pentoside*	0.32 ± 0.02	0.75 ± 0.01	0.77 ± 0.01
2	5.9	330	341	179(100)	Caffeic acid hexoside*	0.33 ± 0.04	0.20 ± 0.01	0.22 ± 0.00
3	8.1	328	353	191(100),179(14),173(6),135(21)	5-*O*-Caffeoylquinic acid*	1.18 ± 0.02	1.29 ± 0.01	1.21 ± 0.01
4	11.3	322	179	135(100)	*trans*-Caffeic acid*	0.33 ± 0.01	0.55 ± 0.01	0.54 ± 0.00
5	16.5	328	473	311(52),293(58),219(32),179(98),149(100),135(66)	Chicoric acid isomer*	3.28 ± 0.07	5.77 ± 0.23	5.95 ± 0.07
6	17.0	330	473	311(46),293(47),219(22),179(100),149(98),135(47)	Chicoric acid isomer*	0.28 ± 0.00	1.09 ± 0.16	0.83 ±0.14
7	19.8	350	593	285(100)	Luteolin *O*-rutinoside**	4.08 ± 0.04	2.20 ± 0.02	1.99 ± 0.04
8	20.9	348	447	285(100)	Luteolin 7-*O*-glucoside**	0.61 ± 0.03	4.26 ± 0.09	4.19 ± 0.09
9	21.5	350	447	285(100)	Luteolin *O*-hexoside**	11.06 ± 0.93	0.59 ± 0.06	0.51 ± 0.05
10	22.5	328	515	353(100),191(85),179(63),173(10),163(8),135(40)	3,5-di-*O*-caffeoylquinic acid*	1.19 ± 0.02	1.24 ± 0.04	0.93 ± 0.00
11	25.1	330	515	353(100),191(42),179(81),173(97),135(28)	4,5-di-*O*-caffeoylquinic acid*	0.02 ± 0.00	0.19 ± 0.00	0.38 ± 0.01
12	26.2	350	489	285(100)	Luteolin *O*-acetylhexoside*	0.23 ± 0.00	0.20 ± 0.01	0.20 ± 0.03
13	34.3	348	285	175(12),151(16),133(23)	Luteolin**	4.29 ± 0.20	2.81 ± 0.24	3.15 ± 0.21
					Total Flavonoids	20.16 ± 1.03[a]	10.07 ± 0.26[b]	10.04 ± 0.36[b]
					Total Phenolic acids	6.94 ± 0.00[c]	11.09 ± 0.11[a]	10.83 ± 0.03[b]
					Total Phenolic compounds	27.22 ± 1.19[a]	21.16 ± 0.37[b]	20.87 ± 0.33[b]

Vegetative parts

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g extract)		
						Methanolic	Infusion	Decoction
1	5.5	330	311	179(100), 135(94)	Caffeic acid pentoside*	3.24 ± 0.10	3.64 ± 0.06	0.67 ± 0.04
2	5.9	330	341	179(28),135(100)	Caffeic acid hexoside*	3.30 ± 0.17	0.23 ± 0.01	0.22 ± 0.00
3	8.1	328	353	191(100),179(14),173(6),135(21)	5-*O*-Caffeoylquinic acid*	0.83 ± 0.04	0.49 ± 0.02	0.31 ± 0.01
4	10.1	328	635	473(90),455(29),341(82),311(3),293(44),219(10),179(100),149(7),135(15)	Chicoric acid hexoside*	1.74 ± 0.16	0.62 ± 0.01	0.25 ± 0.03
5	10.4	358	595	463(40),301(15)	Quercetin *O*-pentosyl hexoside***	0.48 ± 0.00	0.40 ± 0.03	0.07 ± 0.00
6	11.3	322	179	135(100)	*trans*-Caffeic acid*	1.00 ± 0.02	0.46 ± 0.00	0.32 ± 0.00
7	11.8	330	179	135(100)	*cis*-Caffeic acid*	0.60 ± 0.04	0.31 ± 0.01	0.16 ± 0.01
8	13.9	358	595	463(41),301(19)	Quercetin *O*-pentosyl hexoside***	0.34 ± 0.04	0.10 ± 0.01	0.02 ± 0.00
9	15.2	354	667	505(40),463(29),301(10)	Quercetin *O*-hexoside-*O*-acetyl-dihexoside***	0.17 ± 0.03	0.06 ± 0.01	0.02 ± 0.00
10	16.5	328	473	311(55),293(60),219(34),179(100),149(92),135(60)	Chicoric acid isomer*	26.36 ± 0.64	11.93 ± 0.02	2.86 ± 0.19
11	17.4	330	473	311(55),293(47),219(28),179(94),149(100),135(54)	Chicoric acid isomer*	5.68 ± 0.87	1.90 ± 0.03	4.99 ± 0.15
12	19.8	350	593	285(100)	Luteolin *O*-rutinoside**	2.59 ± 0.22	0.60 ± 0.06	0.53 ± 0.01

13	20.3	350	433	301(100)	Quercetin *O*-pentoside***	0.22 ± 0.03	0.06 ± 0.01	0.13 ± 0.00
14	20.9	348	447	327(6), 285(100)	Luteolin 7-*O*-glucoside**	5.67 ± 0.08	1.74 ± 0.03	0.75 ± 0.01
15	22.3	346	505	463(68),301(32)	Quercetin *O*-acetylhexoside***	0.22 ± 0.01	0.08 ± 0.01	0.04 ± 0.00
16	22.5	330	515	353(100),191(75),179(56),173(5),161(6),135(21)	3,5-di-*O*-caffeoylquinic acid*	0.48 ± 0.06	0.11 ± 0.00	0.06 ± 0.00
					Total Flavonoids	9.69 ± 0.23[a]	3.04 ± 0.06[b]	1.74 ± 0.04[c]
					Total Phenolic acids	43.24 ± 0.44[a]	19.70 ± 0.04[b]	9.84 ± 0.05[c]
					Total Phenolic compounds	52.93 ± 0.21[a]	22.74 ± 0.09[b]	11.41 ± 0.07[c]

Calibrations curve used: *- Caffeic acid; **- Luteolin 7-*O*-glucoside; ***- Quercetin 3-*O*-glucoside. The results are expressed in mg per g of methanolic extract or lyophilized infusion and decoction.

Overall, hydroxycinnamic acid derivatives were the main phenolic acids found in both samples, which include caffeic acid derivatives, caffeoylquinic acid derivatives and chicoric acids, the latter being the main compounds found in all the preparations of vegetative parts and in infusion and decoction of flowers. Luteolin derivatives were the only flavonoids identified in flowers, whereas quercetin and luteolin derivatives were present in vegetative parts. The methanolic extracts showed higher amounts of total phenolic compounds than infusions and decoctions. The methanolic extract and the infusion of the vegetative parts showed the highest content in total phenolic compounds, which are correlated with the antioxidant activity displayed by those samples in all the assays: DPPH (R^2=0.9772), reducing power (R^2=0.7362), β-carotene bleaching inhibition (R^2=0.5725) and TBARS (R^2=0.5312). Therefore, the differences observed for antioxidant activity of the samples are related to the amount of phenolic compounds and not with the phenolic compounds profile, which is similar (**Table 36**).

Schütz et al. (2005) also reported chicoric acids as the main phenolic compounds found in dandelion (*Taraxacum officinale*). Indeed, chicoric acids are relevant secondary metabolites in plants of the tribe Cichorieae (family Asteraceae), including genus *Taraxacum* or *Lactuca*, being used for taxonomic purposes (Schütz et al., 2005). Williams et al. (1996) and Gatto et al. (2011), using different extraction and analysis methods, reported similar results on flowers and leaves of *T. officinale*. Shi et al. (2008) identified caffeic acid as one of the major compounds in *T. mongolicum*.

In conclusion, flowers of wild dandelion gave higher content of total sugars (despite the lack of trehalose and raffinose), tocopherols (mainly α-isoform) and flavonoids (mainly luteolin *O*-hexoside and luteolin) than vegetative parts. In contrast, the latter showed higher content of proteins, ash, organic acids, PUFA (mainly linoleic acid) and phenolic acids (caffeic acid derivatives and especially chicoric acid), lower levels of total fat and energy, and better PUFA/MUFA (above 0.45) and n6/n3 (lower than 4.0) ratios. In general, vegetative parts of dandelion gave also higher antioxidant activity, which could be related to its higher content in phenolic acids (R^2=0.9964, 0.8444, 0.4969 and 0.5542 for DPPH, reducing power, β-carotene bleaching inhibition and TBARS assays, respectively). Particularly, vegetative parts decoction showed the highest DPPH scavenging activity and reducing power, and its methanolic extract revealed the highest lipid peroxidation inhibition (TBARS assay).

As far as we know, this is a groundbreaking study on the nutraceutical composition, bioactivity and phenolic profile of flowers and vegetative parts of wild dandelion (*ie*, *Taraxacum* sect. *Ruderalia*). This study also demonstrates that wild plants like *Taraxacum*, although not being a common nutritional reference, can be used in an alimentary base as a source of bioactive compounds, namely antioxidants.

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3.4.1.4. References

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3.5. Estudos de bioacessibilidade de minerais

Neste sub-capítulo apresenta-se um estudo da bioacessibilidade de minerais provenientes de *Achillea millefolium* L., *Laurus nobilis* L. e *Taraxacum* sect. *Ruderalia* e respetivas infusões. Também se apresenta o conteúdo em folatos das respetivas amostras.

3.5.1. Minerais e folatos em plantas secas vs infusões: avaliação da dinâmica de absorção de minerais em membranas de diálise simulando uma digestão *in vitro*.

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Minerals and vitamin B_9 in dried plants *vs.* infusions: assessing absorption dynamics of minerals by membrane dialysis tandem *in vitro* digestion

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Running title: Minerals and vitamin B_9 in dried plants vs. infusions: extractability and bioacessability

Abstract

Vitamins and mineral elements are among the most important phytochemicals due to their important role in the maintenance of human health. Despite these components had already been studied in different plant species, their full characterization in several wild species is still scarce. In addition, the knowledge regarding the *in vivo* effects of phytochemicals, particularly their bioaccessibility, is still scarce. Accordingly, a membrane dialysis process was used to simulate gastrointestinal conditions in order to assess the potential bioaccessibility of mineral elements in different preparations of *Achillea millefolium* (yarrow), *Laurus nobilis* (laurel) and *Taraxacum* sect. *Ruderalia* (dandelion). The retention/passage dynamics was evaluated using a cellulose membrane with 34 mm pore.

Dandelion showed the highest levels of all studied mineral elements (except zinc) independently of the used formulations (dried plant or infusion), but yarrow was the only species yielding minerals after the dialysis step, either in dried form, or as infusion. In fact, the ability of each evaluated element to cross the dialysis membrane showed significant differences, being also highly dependent on the plant species. Regarding the potential use of these plants as complementary vitamin B_9 sources, the detected values were much lower in the infusions, most likely due to the thermolability effect.

Keywords: Vitamin B_9; Minerals; Infusions; Wild plants

3.5.1.1. Introduction

The interest for traditionally used plants is rising, since they are considered a valuable and reliable source of natural compounds with recognised health effects. Among those compounds, the study of vitamins and mineral elements is crucial, due to their important role in the maintenance of human health; in fact, the lack of vitamins can cause a number of diseases, and mineral trace elements have essential biochemical functions such as the activation of chemical components present in the organism (Rihawy et al., 2010). The possible applications of plants should be complemented by a complete chemical characterization (Leśniewicz et al., 2006). Despite the high number of scientific publications profiling chemical compounds in plants, some wild species are still lacking for comprehensive studies. *Achillea millefolium* L. (yarrow, Asteraceae), *Laurus nobilis* L. (bay leaves, Laureacea) and *Taraxacum* sect. *Ruderalia* (dandelion, Asteraceae) were scarcely studied for their mineral profile and vitamin B_9 composition, making them good candidates for this type of profiling studies.

Vitamin B_9 (folic acid/folates) is an important cofactor of many biochemical reactions in cells. The absence of this vitamin would lead to non-cell division, anaemia, cardiovascular disease and neural tube defects in infants. Common food sources of vitamin B_9 are vegetables, bread and cereals, which may contain various forms of folate depending on food processing and storage. In food, folates are naturally presented as polyglutamates (PteGlun), mainly as mono-, penta- and hexaglutamates (Scott et al., 2000), being the monoglutamate form absorbed in the intestinal tube (Scott, 1999) and further converted to tetrahydrofolate (the most bioactive form of this vitamin) (Bailey & Ayling, 2009).

Microelements such as iron (Fe), copper (Cu), manganese (Mn) and zinc (Zn) represent a group correlated with the prevention of cardiovascular diseases, and some of them display also important biological functions such as osmoprotection (Fe), mitochondrial respiration (Cu), and energy production and maintenance of structural integrity of

biomembranes (Zn) (Hänsch & Mendel, 2009). These elements, which are required by the body in low amounts, can be obtained (together with numerous organic compounds) in the infusions of medicinal plants, subsequently leading to different physiologic functions, toxicity and absorption rates (Mutaftchiev, 2001; Özcan, 2004). Macroelements such as calcium (Ca), phosphorus (P), magnesium (Mg), potassium (K) and sodium (Na) serve as structural elements of the tissues and modulate the metabolism and acid-base balance, being present in the body in higher amounts than microelements (Leśniewicz et al., 2006; Özcan, 2004). Within the same species, the concentration of micro and macroelements in plants is conditioned by geochemical characteristics, rainfall and agricultural practices (Łozak et al., 2002; Konieczyński & Wesołowski, 2007).

Many exogenous (food matrix and compound structure) and endogenous (active transport, metabolism and excretion in the human body) factors affect the entrance of compounds in the lumen and therefore its bioavailability. As a part of the concept of bioavailability, bioaccessibility is defined as the amount of a food constituent that is present in the gut as a consequence of its release from the solid food matrix, and may be able to pass through the intestinal barrier and be potentially bioavailable (Saura-Calixto et al., 2007). *In vitro* gastrointestinal models provide a very useful methodology to screen food ingredients (*e.g.*, minerals, vitamins, phenolic compounds, among others) for their bioavailability. These system provides a great amount of results in a short period of time, allowing the study of matrices with different compositions and structures, simultaneously overcoming the complexity of *in vivo* studies (Hur et al., 2011).

The content of mineral elements was already determined by atomic absorption spectroscopy methods in *A. millefolium* (Chizzola et al., 2003; Konieczyński & Wesołowski, 2007; Divrikli et al., 2006), *L. nobilis* (Özcan, 2004; Divrikli et al., 2006; Sekeroglu et al., 2008; Zengin et al., 2008) and *Taraxacum obovatum* (Willd.) DC. basal leaves (García-Herrera et al., 2014) samples from different locations. Nevertheless, to our knowledge, there are no reports of the content of vitamin B_9 in yarrow or bay leaves. A particular species of dandelion, *Taraxacum obovatum* (Willd.) DC., was previously studied for the vitamin B_9 content in its basal leaves (Morales et al., 2014). Nevertheless, to our knowledge, there are no studies on the vitamin B_9 content of yarrow and laurel, nor on the *in vitro* bioaccessibility of mineral elements from the plants studied herein. Therefore, the main objective of the present work was to characterize vitamin B_9 and minerals profile in dried material and infusions of wild samples of *A. millefolium*, *L. nobilis* and *Taraxacum* sect. *Ruderalia*. Furthermore, an *in vitro* gastrointestinal model was applied to provide a preliminary study of mineral elements bioaccessibility in these food matrices.

3.5.1.2. Materials and methods

Samples and infusions preparation

The wild samples of yarrow (inflorescences and upper leaves), laurel (leaves; before flowering) and the vegetative parts of wild *Taraxacum* sect. Ruderalia were collected in Bragança (Portugal). Voucher specimens of yarrow (nº 9623 BRESA), laurel (nº 9634 BRESA) and dandelion (nº 9686) were deposited at the Herbarium of the Escola Superior Agrária de Bragança (BRESA) (Dias et al., 2013; Dias et al., 2014a; Dias et al., 2014b). Morphological key characters from the Flora Iberica (Castroviejo, 1986-2012) were used for plant identification. The wild samples were lyophilized (FreeZone 4.5, Labconco, Kansas, USA) and stored at 4ºC until analysis.

The infusions were prepared according to the traditional procedure used to prepare tea (1 bag with ~1 g dry material, and 1 teapot with ~200 mL); therefore, each sample (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure. The obtained infusions were frozen, lyophilized and stored at -6 ºC until analysis.

Standards and reagents

Micro (Fe, Cu, Mn and Zn) and macroelements (Ca, Mg, Na and K) standards (> 99% purity), as well $LaCl_2$ and CsCl (> 99% purity) were purchased from Merck (Darmstadt, Germany). Standards of 5-CH_3-H_4folate monoglutamate (ref. 16252; Schircks Laboratories, Jona, Switzerland) and pteroyl diglutamic acid (ref. 16235; Schircks Laboratories, Jona, Switzerland), pancreatic chicken homogenate (Pel Freeze, Arkansas), rat serum, $NaBH_4$, formaldehyde and octanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile fluorescence grade was bought from Fisher Scientific (Madrid, Spain). All other general laboratory reagents were purchased from Panreac Química S.L.U. (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Vitamin B_9 (folic acid/folates)

Vitamin B_9 content was determined according to the methodology previously described by Morales et al. (2015), using HPLC-FL system, consisted of a Beta 10 (Ecom, Prague, Czech Republic) gradient pump with Gastorr Degasser HPLC Four Channel BR-14 (Triad Scientific, New Jersey, USA) as degassing device, joined to an AS-1555 automatic injector (Jasco, Easton, MD, USA), and to a FP-2020 Plus Fluorescence detector (Jasco, Easton, MD, USA) with RP 18 endcapped Lichrospher 100 column (Merck, Darmstadt, Germany; 250 × 5 mm; 5 µm). Quantification was performed by comparison of the area of

the peaks recorded with calibration curves obtained from commercial standards (5-CH_3-H_4folate mono and diglutamate), and expressed as total folates (from the sum of both compounds) per 100 g plant (dw) or per 100 mL infusion.

Chromatographic parameters, namely limit of detection (LOD), limit of quantification (LOQ), linearity, recovery, repeatability and reproducibility were accepted as previously assessed (Morales et al., 2015).

Mineral and trace elements content

Mineral elements analysis was performed according to the method 930.05 of AOAC procedures for ash obtention, and then following the methodology previously described by Fernández-Ruiz, Olives, Cámara, Sánchez-Mata & Torija (2011). All measurements were performed in atomic absorption spectroscopy (AAS) with air/acetylene flame in Analyst 200 Perkin Elmer equipment (Perkin Elmer, Waltham, MA, USA), comparing absorbance responses with > 99.9% purity analytical standard solutions for AAS made with $Fe(NO3)_3$, $Cu(NO_3)_2$, Mn $(NO_3)_2$, Zn $(NO_3)_2$, NaCl, KCl, $CaCO_3$ and Mg band. Limit of detection (LOD), limit of quantification (LOQ), linearity, recovery, repeatability and reproducibility were accepted as previously assessed (Sanchez-Mata, 2000).

In vitro gastrointestinal model (dialysis)

The *in vitro* model applied consisted of an initial simulation phase of intraluminal digestion, followed by an intestinal absorption using a dialysis model (Ramírez-Moreno et al., 2011). Thus, minerals bioaccessibility was estimated using 25 mL of aqueous solutions prepared from dry material (20 mg/mL) or lyophilized infusion (20 µg/mL), Gastric digestion was simulated, adjusting the pH of each sample to 2, adding 150 µL of a pepsin solution (40 mg/mL of HCl 0.1M), and incubating the mixture in a water bath at 37^{o}C for 2 h with stirring (60 osc/min). The intestinal processes were then simulated, adding to the digested product a pancreatin/bile solution (5/25 mg of pancreatin/bile per 1 mL of 0.1M $NaHCO_3$). The mixture was then transferred to dialysis membranes (Medicell 7000/2, width 34 mm, 7000 MW cut off), previously boiled in distilled water for 15 min. The dialysis membranes/mixture was then placed into a flask containing 250 mL of $NaHCO_3$ pH 7.5 and incubated in a water bath at room temperature for 3 h with stirring (60 osc/min). After dialysis, the obtained final solution of $NaHCO_3$ pH 7.5 was frozen and lyophilized for further assays.

Statistical analysis

For each plant material, three samples were used and all the assays were carried out in triplicate. When evaluating macroelements bioaccessibility, the results were expressed as

mean values±standard deviation (SD) and differences were analysed using a *t*-student test, since there were fewer than 3 groups.

Regarding the evaluation of the effects of plant species (*A. millefolium*, *L. nobilis* or *T.* sect. *Ruderalia*) and formulation (dried plant or infusion), an analysis of variance (ANOVA) with type III sums of squares was performed using the Repeated Measures Analysis procedure of the General Linear Model. Since the independence of variables could not be assumed, it was need to verify the sphericity criterion, which evaluates if the correlation between treatments is the same, assuming that variances in the differences among conditions are equal. Sphericity was evaluated trough the Mauchly's test; every time the sphericity assumption was violated, the Greenhouse-Geisser correction was applied.

All the statistical analyses were carried out using SPSS v. 22.0 program (IBM Corp., Armonk, NY, USA).

3.5.1.3. Results and discussion

In the evaluated parameters, it was intended to verify the effects of plant species, independently of the used formulation, and the differences among formulations, regardless of the plant species. Accordingly, results were compared by a 2-way ANOVA, following the generalized linear model coupled to the repeated measures analysis technique. In this analysis, it is important to check for the homogeneity of variances in the measures done for each of the factors' levels. Since the independence of variables cannot be assumed, the former requisite was evaluated by the Mauchly's sphericity test.

The results obtained for the infusions (which were prepared using ~1 g of dried plant material) were converted to be expressed in 100 g of dried plant basis to allow their direct comparison with those obtained from the direct analysis of the dried plant.

Effects on microelements

The results for iron, copper, manganese and zinc are given in **Table 37**. The evaluated factors, plant species (PS) and formulation (F) showed a significant interaction (PS×F) in all cases, indicating that the yields in microelements that can be obtained from the dried plant or its infusion are highly dependent of the used plant species (and *vice-versa*). This occurrence hampers the possibility of indicating the best plant (independently of the formulation) and the formulation with highest suitability to be used for microelements obtention (independently of the plant species). Nevertheless, the effect of each individual factor *per se* was also significant in all cases, allowing to indicate specific trends: dandelion seemed to be the best source of iron (29.3 mg/100 g dw), copper (1.87 mg/100 g dw) and manganese (5.1 mg/100 g dw), while laurel gave the highest contents in zinc (9.1 mg/100 g

dw). The results obtained for yarrow samples are in the same range as those quantified previously, despite the higher zinc levels (6.61 mg/100 g) in Polish samples (Konieczyński & Wesołowski, 2007) and the lower levels of iron (2.65 mg/100 g). On the other hand, Divrikli et al. (2006) reported higher levels of iron and copper (31.67 and 1.76 mg/ 100 g, respectively), but similar concentrations of manganese and zinc (4.23 and 2.54 mg/ 100 g, respectively). In a study conducted in Turkish laurel samples, the levels of copper, iron and manganese were also detected in higher amounts (Divrikli et al. 2006; Özcan, 2004; Zengin et al., 2008).

Among the formulations, using the powdered plant directly, instead of its infusion, would be the right option to maximize the yield in microelements. In fact, the extraction percentages for each microelement were quite dissimilar Mn<<<Zn<Cu<<Fe.

Table 37. Composition in micro-elements of powdered material and infusions (mg/100 g) of the studied wild samples. Results are presented as estimated marginal mean±standard error

		Micro-elements			
		Fe	Cu	Mn	Zn
Plant species	Yarrow	4.8±0.1	0.79±0.01	3.8±0.1	2.3±0.1
	Laurel	5.9±0.1	1.22±0.03	1.2±0.1	9.1±0.1
	Dandelion	29.3±0.5	1.87±0.01	5.1±0.1	4.8±0.1
Mauchly's test of sphericity (*p*-value)		0.105 (<0.001)	0.496 (0.086)	0.132 (0.001)	0.062 (<0.001)
p-value[a]		<0.001	<0.001	<0.001	<0.001
Formulation	Powder	17.0±0.4	2.06±0.02	6.5±0.1	9.0±0.1
	Infusion	9.7±0.2	0.52±0.01	0.17±0.01	1.8±0.1
Mauchly's test of sphericity		1.000	1.000	1.000	1.000
p-value[a]		<0.001	<0.001	<0.001	<0.001
PS×F interaction					
Mauchly's test of sphericity (*p*-value)		0.024 (<0.001)	0.361 (0.028)	0.248 (0.008)	0.097 (<0.001)
p-value[a]		<0.001	<0.001	<0.001	<0.001

[a]Significance value for the tests of between subjects effects. When sphericity assumption was not met (p<0.05), the *p*-value was obtained from the Greenhouse-Geisser correction.

Effects on macro-elements

The results for calcium, magnesium and potassium are given in **Table 38**. The elements detected in highest amount in the samples of yarrow, laurel and dandelion were potassium, calcium and magnesium. In line with the observed for microelements, the interaction (PS×F) was significant ($p < 0.05$) in all cases. Nevertheless, the significant differences found for each factor (except for the effect of the formulation on the magnesium levels) allowed the identification of some overall trends. Dandelion showed the highest values in macro-elements (Ca: 882 mg/ 100 g dw, Mg: 223 mg/ 100 g dw; K: 2851 mg/ 100 g dw), while laurel gave the lowest (Ca: 283 mg/ 100 g dw, Mg: 88 mg/ 100 g dw; K: 484 mg/ 100 g dw), independently of their quantification in dried samples or their infusions. Chizzola

et al., (2003) described lower values of mineral elements in a yarrow sample from Austria, while Özcan (2004) reported higher calcium content (1076.1 mg/100 g), but similar values for potassium (493.7 mg/ 100 g dw) in Turkish laurel samples. The powdered plants allowed higher macro-elements yields when compared to the samples prepared by infusion, but the extraction yields (particularly for magnesium and calcium) were higher than those achieved for the microelements. The concentration of mineral elements in infusions strongly depends on the type of bound formed with the plant cells, but also on its solubility in the solvent used for the extraction. In addition, the heat treatment may also have some influence in the final concentration of specific minerals in the infusions, since it can influence the extraction yield of these elements, breaking its connection with cell constituents (Pytlakowska et al., 2012). Therefore, the differences found in the released percentage of minerals in the infusions could be explained by the obvious biological and botanical differences existing in the tissues of each one of the plants, which could modulate the extraction of mineral elements from the plant cells. When comparing the results obtained in the powdered plants and in the infusions, it might be concluded that manganese and potassium were, respectively, the micro- and macro-element that were most retained by the plants during the infusion process. In general, these results indicate higher extraction efficiency of mineral elements to infusions than the obtained by Zengin et al. (2008), despite the different solid to solvent ratios (1:200 in our case, 1:20 in the research reported by Zengin et al. (2008).

Table 38. Composition in macro-elements of dried material and infusions (mg/100 g) of the studied wild samples. Results are presented as estimated marginal mean±standard error.

		Macro-elements		
		Ca	Mg	K
Plant specie	Yarrow	395±5	172±5	1267±10
	Laurel	283±2	88±1	484±7
	Dandelion	882±8	223±2	2851±52
Mauchly's test of sphericity (*p*-value)		0.141 (0.001)	0.221 (0.005)	0.193 (0.003)
p-value[a]		<0.001	<0.001	<0.001
Formulation	Powder	564±5	167±4	1889±36
	Infusion	476±3	156±2	1178±8
Mauchly's test of sphericity		1.000	1.000	1.000
p-value[a]		<0.001	0.051	<0.001
PS×F interaction				
Mauchly's test of sphericity (*p*-value)		0.893 (0.673)	0.612 (0.180)	0.548 (0.122)
p-value[a]		<0.001	<0.001	<0.001

[a]Significance value for the tests of between subjects effects. When sphericity assumption was not met (p<0.05), the *p*-value was obtained from the Greenhouse-Geisser correction.

Vitamin B_9 in dry plant and infusions

Once again, the differences among the yields obtained using dried plant or its infusion depend on the assayed plant species (*i.e.*, the interaction PS×F was significant, **Figure 18**). Regardless of the formulation, the highest amounts of vitamin B_9 were quantified in yarrow (257 µg/100 g dw), followed by dandelion (91 µg/100 g dw) and laurel, in which this vitamin was nearly absent (0.082 µg/100 g dw). In fact, the potential of vegetables to act as sources of vitamin B_9 varies greatly; some examples such as asparagus, spinach and okra are considered excellent, but others like as celery, kale, broccoli and even lettuce, contain very limited levels of this vitamin (Suitor & Bailey, 2000).

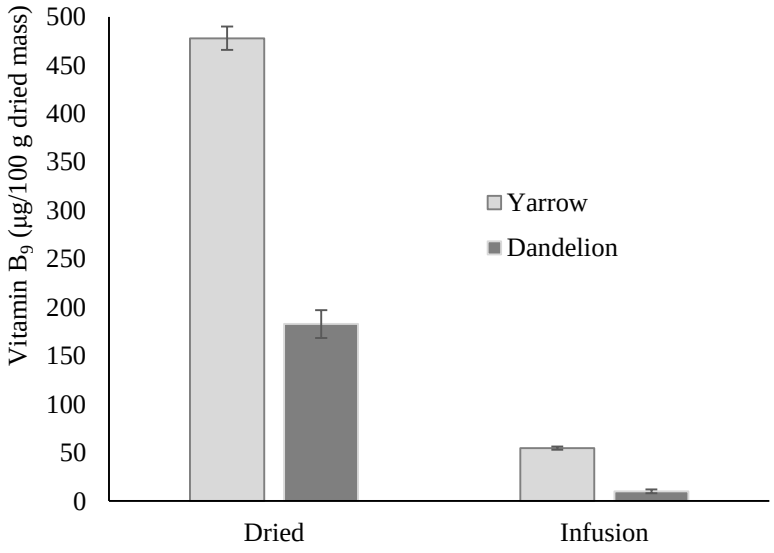

Figure 18. Estimated marginal mean plots representing the effect of plant species and formulation on vitamin B_9 levels. Bars corresponding to laurel samples were supressed due to their low magnitude (vitamin B_9 was nearly absent in laurel).

When comparing the dried plants with the corresponding infusions, a ~10-fold difference was detected (powder: 210 µg/100 g dw; infusion 22 µg/100 g dw). This can be explained by the fact that vitamin B_9 has high solubility and reactivity, being susceptible to degradation in many processing steps, including the high temperatures used for the infusions preparation (Scott et al., 2000). Furthermore, the potential retention of the vitamin B_9 native form by the vegetal matrices, due to its interaction with other plant constituents that effectively could influence its bioavailability, is a well-known fact, which might also explain this difference.

The vitamin B_9 levels detected in yarrow and dandelion might offer new possible applications for these plant species. It has been stated that a rich vitamin B_9 diet reduces the risk of chronic diseases, such as cardiovascular problems. Several international organizations, and particularly the Food and Nutrition Board (Trumbo et al., 2002), have

Recommended Dietary Allowance (RDA) of 400 µg of folic acid, with particular relevancy among pregnant women (Krawinkel et al., 2014). Moreover, according to the Regulation (EC) No. 1169/ 2011 (Regulation (EC) No. 1169/ 2011) of the European Parliament and of the Council, of 25 October 2011, on the provision of food information to consumers, it is necessary an intake of at least 7.5 and 15% of de NRV (Nutritional References Values) of this vitamin (200 µg/day) to consider the studied infusions and plants as "sources of vitamin B_9". The detected levels of vitamin B_9, despite relevant among natural sources, did not allow considering these plants as the sole daily source of this vitamin.

Bioaccessibility studies

After *in vitro* digestion only a few minerals were detected in all plant samples as it can be seen in **Figure 19**. The majority of mineral found were macroelements (calcium, magnesium and potassium), despite the presence of low amounts of manganese. *A. millefolium* was the only plant that presented dialyzable minerals in both formulations, dried plant and infusion. Potassium and manganese were detected in the dried plant of yarrow (433.31mg/100g and 0.14mg/100 g, respectively, data no shown), which represented 26% and 2%, respectively of minerals that passed through the dialysis membrane. In the yarrow infusion, the only detected element was calcium (2.25 mg/100 mL, data not shown) that reached 76% of mineral passing through the membrane. *L. nobilis* only showed dialyzable minerals in the infusion form, particularly potassium and calcium (1.33 mg/ 100 mL in both minerals), corresponding to 48% of mineral that passed after dialysis. On the other hand, no micro or macroelements were detected in laurel dried material after *in vitro* digestion. Probably, these elements were below the limit of detection of the AAS technique (usually limited to the ppm range).

In *T*. sect *Ruderalia* the dialyzable minerals were only detected in the dried plant. In this case, magnesium, calcium and manganese were not completely retained, yielding 5% (0.9 mg/100 g), 25% (214.7 mg/100 g) and 4% (7.9 mg/100 g) of their global amounts.

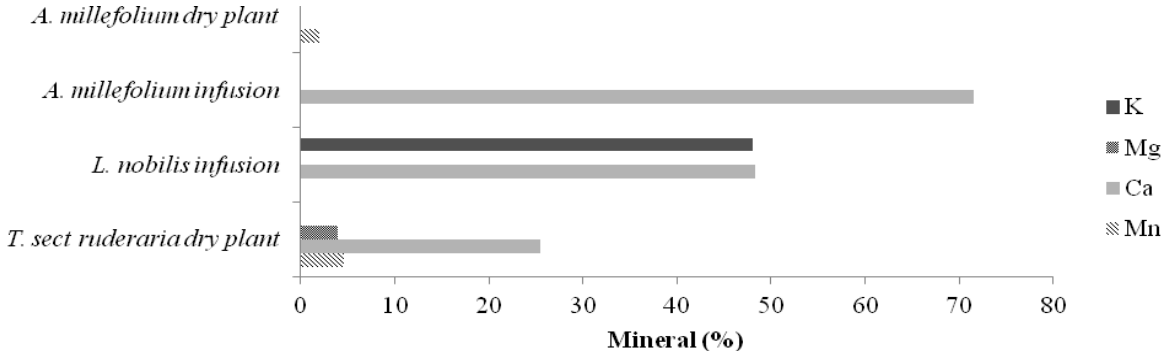

Figure 19. Macro and microelements bioaccessibility percentages in *Achillea millefolium* L., *Laurus nobilis* L. and *Taraxacum* sect. *Ruderalia* infusions, after *in vitro* gastrointestinal digestion.

3.5.1.4. Conclusion

Dandelion showed the highest levels of all studied micro (except zinc, which showed the highest content in laurel) and macroelements, independently of the used formulation. On the other hand, yarrow gave the highest content in vitamin B9. Dried plants, as expected, allowed higher contents in all analytes when compared to the corresponding infusions; nevertheless, the extraction yields for mineral elements varied greatly, being higher for the macroelements: Mg>Ca>K>Fe>Cu>Zn>Mn. The levels of vitamin B_9 were much lower in the infusions, most likely due to the degradation induced by using boiling water.

Regarding the bioaccessibility, the elements with best performance in the dialysis process were calcium and potassium.

Overall with this preliminary study, the studied plant species, especially if used directly in the dried form, might be considered in the development of novel food formulations.

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Conflict of interest

The authors declare that they have no conflict of interest.

3.5.1.5. References

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4.

Utilização da cultura *in vitro* para estimular a produção de bioativos em *Fragaria vesca* L.

Neste capítulo apresenta-se a cultura *in vitro* como ferramenta biotecnológica para a produção de compostos fenólicos de forma sustentável. Descreve-se o estabelecimento de uma cultura *in vitro* a partir do fruto silvestre de *Fragaria vesca* L. e a sua caracterização nutricional, química e propriedades antioxidantes.

4.1. Partes vegetativas de *Fragaria vesca* L. silvestre: será a cultura *in vitro* capaz de melhorar os compostos nutricionais e bioativos

Submitted

Vegetative parts of wild *Fragaria vesca* L.: is *in vitro* culture able to enhance nutritional and bioactive compounds

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Abstract

In vitro culture emerges as a sustainable way to produce bioactives for further applicability in the food industry. Herein, vegetative parts of *Fragaria vesca* L. (wild strawberry) obtained by in vitro culture were analyzed regarding nutritional and phytochemical compounds, as also antioxidant activity. These samples proved to have higher protein content, polyunsaturated fatty acids, soluble sugars, organic acids (including ascorbic acid) and tocopherols (mainly α-tocopherol) than wild grown *F. vesca*, being also detected different phenolic compounds. The antioxidant activity of hydromethanolic extracts could be correlated with the content of different phenolic groups and other compounds (sugars and organic acids). It was demonstrated that in vitro culture could enhance nutritional and bioactive compounds of *Fragaria vesca* L. plants, being a very interesting biotechnological tool to obtain them for further food applicability.

Keywords: *Fragaria vesca* L.; *in vitro* culture; nutritional value; phenolic compounds

4.1.1. Introduction

Wild strawberry (*Fragaria vesca* L., Rosaceae family) can be commonly found in Europe, Japan, North America and Canada, growing wildly in mountain zones, forests, slopes and roadsides (Castroviejo et al., 1998). It is mainly appreciated for its sweet small fruits, however the vegetative parts have been described as important sources of macro and micronutrients and also phenolic compounds (procyanidins, ellagic acid and hydroxycinnamoyl derivatives) with strong antioxidant activity (Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Morales, et al., 2015; Simirgiotis & Schmeda-Hirschmann, 2010). A daily basis consumption of vegetative parts from *F. vesca* could provide tonic, antiseptic and detoxifying properties (Neves, Matos, Moutinho, Queiroz, & Gomes, 2009; Sõukand & Kalle, 2013). Furthermore, its decoctions and infusions have been traditionally used to treat urinary tract infections and hypertension, presenting also antidiarrheal and anticoagulant activity (Camejo-Rodrigues, Ascensão, Bonet, & Vallès, 2003; Özüdoru, Akaydin, Erik, & Yesilada, 2011; Pawlaczyk, Czerchawski, Pilecki, Lamer-Zarawska, & Gancarz, 2009; Savo, Giulia, Maria, & David, 2011).

The growing demand for natural products that complement their nutritional role with additional functional properties requires innovation in the ways to obtain these products, in order to protect wild populations from where they are obtained, and also to avoid competing directly with crops that are used for food (Godfray et al., 2012).

Plant tissue culture appears as a valuable technique to produce secondary metabolites, being an ecological and sustainable alternative for the production of endangered species (by overexploitation), but also to obtain bioactive extracts and compounds that can be further applied in pharmaceutical/medical field or in the food industry. Indeed, this approach has been endorsed by FAO as safe for compounds production for food applications (Dias, Sousa, Alves, & Ferreira, 2016). Regardless of the climate or geographic conditions, this technique allows a continuous production of natural compounds under a very restricted controlled regime (Anand, 2010; Karuppusamy, 2009).

The nutritional value and chemical profile of vegetative parts of *F. vesca* was previously reported by our research group (Dias, Barros, Morales, et al. 2015). The presence of sugars and organic acids was also described in its fruits (Doumett et al., 2011; Ornelas-Paz et al., 2013), while phenolic compounds and related bioactive properties were reported in different parts (fruits, leaves and roots) (Clifford, 2000; da Silva Pinto, Lajolo, & Genovese, 2008; Del Bubba et al., 2012; Dias et al., 2016; Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, Santos-Buelga, & Ferreira, 2015; Gasperotti et al., 2013; Simirgiotis & Schmeda-Hirschmann, 2010; Sun, Liu, Yang, Slovin, & Chen, 2014; Zheng, Wang, Wang, &

Zheng, 2007). These compounds were also described in *F. vesca* obtained from *in vitro* culture, after optimization of growth conditions (concentration of plant regulators and regeneration enhancers) (Yildirim & Turker, 2014). Nevertheless and to the author's best knowledge, no other components have been studied.

In the present work, vegetative parts of *Fragaria vesca* L. were obtained by *in vitro* culture and further characterized in terms of macronutrients, fatty acids, soluble sugars, organic acids, tocopherols and phenolic compounds, as also regarding the antioxidant activity. The studies were carried out with lyophilized material, hydromethanolic extracts and aqueous consumption forms (infusions and decoctions).

4.1.2. Materials and methods

Standards and Reagents

Acetonitrile (99.9%), n-hexane (95%) and ethyl acetate (99.8%) were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Acetonitrile fluorescence grade was bought from Fisher Scientific (Madrid, Spain). Formic acid was purchased from Prolabo (WWR International, France). Fatty acids methyl ester (FAME) reference standard mixture (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as well as other individual fatty acid methyl ester isomers, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), L-ascorbic acid, tocopherol, sugar and organic acid standards were purchased from Sigma-Aldrich. Phenolic standards were from Extrasynthèse (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other general laboratory reagents were purchased from Panreac Química S.L.U. (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Samples and establishment of an in vitro *culture of Fragaria vesca L.*

The samples of wild *Fragaria vesca* L. fruits were collected in Serra da Nogueira, Bragança, North-eastern Portugal, in July 2013. The establishment of the *in vitro* culture was achieved by using the wild fruits with the seeds. The fruits were washed with tap water and sterilized with bleach and detergent for 5 min under agitation, washed with sterilized water and inoculated in a basic medium for seed germination with water and agar (0.9%) and kept in the dark until germination (Figure 20. **Establishment of an in vitro culture of wild Fragaria vesca L. from its fruits (A); Detachment of fruit seedlings (B) and *in vitro* growth of aerial parts (C).).****Figure 20A**). The seedlings were then detached from the fruit (**Figure 20B**) and placed in a modified culture medium (Murashige & Skoog, 1962) supplied with macronutrients, l mg/L thiamine, 1 mg/L nicotinic acid, 1 mg/L pyridoxine, 2% sucrose, 0.5 mg/L BAP (benzylaminopurine) and 0.5 mg/L IBA

(indole-3-butyric acid). The pH culture medium was adjusted to 5.7 before autoclaving. The culture conditions were Tmin [16-18] ºC, Tmax [24-26] ºC with a photoperiod of 16/8 h (light/dark) supplied by light-bulbs Silvana day light (Phillips, Amsterdam, Netherlands). The plants were kept in the same culture conditions and subcultured every month (**Figure 20C**), collecting the aerial partsts and keeping the roots for further growth. The collected aerial partswere stored at -20 ºC, lyophilized and reduced to a fine powder for further analysis.

A **B** **C**

Figure 20. Establishment of an in vitro culture of wild Fragaria vesca L. from its fruits (A); Detachment of fruit seedlings (B) and *in vitro* growth of aerial parts (C).).

Preparation of the aqueous consumption forms

For infusions preparation, the lyophilized plant material (500 mg) was added to 100 mL of boiling distilled water (pH 6.6) at 100 ºC, left to stand at room temperature for 5 min and then filtered under reduced pressure (0.22 µm).

For decoctions preparation, the lyophilized plant material (500 mg) was added to 100 mL of distilled water, heated and boiled for 5 min. The mixture was left to stand for 5 min and then filtered under reduced pressure. The extracts obtained by infusion and decoction were lyophilized for further analysis of phenolic compounds and antioxidant activity.

Nutritional value of the lyophilized plant material

Proximate composition

The lyophilized plant material was analyzed for proteins, fat, carbohydrates and ash according to the AOAC procedures (AOAC, 2005). The crude protein content (N×6.25) was estimated by the macro-Kjeldahl method (AOAC, 991.02); the crude fat (AOAC, 989.05) was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content (AOAC, 935.42) was determined by incineration at 550±15 ^{o}C; Total carbohydrates were calculated by difference. Total energy was calculated according to the following equation: Energy (kcal/100 g) = 4 × (g proteins + g carbohydrates) + 9 × (g fat), according to the (Regulation (EC) No 1169/2011, 2011).

Fatty acids

Fatty acids were determined in the lyophilized plant material, after a trans-esterification process as previously described (Barros et al., 2013). The fatty acids profile was analysed using a gas-liquid chromatographer (DANI model GC 1000 instrument, Contone, Switzerland) equipped with a split/splitless injector and a flame ionization detection (GC-FID, 260 ºC) and a Macherey–Nagel (Düren, Germany) column (0.5 g/kg cyanopropyl-methyl-0.5 g/kg phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 µm df). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30 ºC/min ramp to 125 ºC, 5 ºC/min ramp to 160 ºC, 20 ºC/ min ramp to 180 ºC, 3 ºC/min ramp to 200 ºC, 20 ºC/min ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (61000 Pa), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC). The identification was made by comparing the relative retention times of FAME (Fatty Acid Methyl Esters) peaks of the samples with commercial standards. The results were recorded and processed using Clarity 4.0.1.7 Software (DataApex, Prague, Czech Republic) and expressed in relative percentage of each fatty acid.

Chemical characterization of the lyophilized plant material and aqueous consumption forms

Soluble sugars

The extraction of soluble sugars from the lyophilized plant material was carried out following the procedure described by Barros et al. (2013), while for the aqueous preparations the analysis was carried out directly. Soluble sugars were determined by high performance liquid chromatography equipment consisting of an integrated system with a pump (Knauer,

Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000) and auto-sampler (AS-2057 Jasco, Easton, MD, USA), coupled to a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany), as previously described (Barros et al., 2013). The chromatographic separation was achieved with a Eurospher 100-5 NH2 column (5 µm, 250 × 4.6 mm i.d., Knauer) operating at 35 ºC (7971 R Grace oven). The mobile phase was acetonitrile (700 mL/L)/deionized water (300 mL/L), at a flow rate of 1 mL/min. The identification was carried out by chromatographic comparisons of the relative retention times of sample peaks with authentic standards, while the quantification was performed using the internal standard (melezitose) method and by using calibration curves obtained from the commercial standards of each compounds. The results were expressed in g per 100 g of dry weight or in mg per 100 mL in the case of infusions and decoctions.

Organic acids

The extraction of organic acids from the lyophilized plant material was carried out following the procedure described by Barros et al. (2013), while for the aqueous preparations the analysis was carried out directly. Vitamin C and other organic acids were determined by ultra-fast liquid chromatography coupled to photodiode array detection (UFLC-PDA; Shimadzu Coperation, Kyoto, Japan) and following a procedure previously described (Barros et al., 2013). Separation was achieved on a SphereClone (Phenomenex) reverse phase C_{18} column (5 µm, 250 × 4.6 mm) thermostatted at 35 ºC. The elution was performed with sulphuric acid 3.6 mmol/L using a flow rate of 0.8 mL/min. The quantification was performed by comparison of the area of the peaks recorded at 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight or in mg per 100 mL in the case of infusions and decoctions.

Tocopherols

The extraction of tocopherols from the lyophilized plant material was carried out following the procedure described by Barros et al. (2013), while for the aqueous preparations the analysis was carried out directly using HPLC coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II normal-phase column (5 µm, 250 × 4.6 mm i.d., YMC Waters), operating at 35 °C. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, *v/v*) at a flow rate of 1 mL/min. The identification was performed by chromatographic comparisons with authentic standards,

while the quantification was based on the fluorescence signal response of each standard, using the internal standard (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in μg per 100 g of dry weight or in μg per 100 mL in the case of infusions and decoctions.

Bioactivity of hydromethanolic extracts and aqueous consumption forms

Preparation of the hydromethanolic extracts

The lyophilized plant material (1 g) was submitted to an extraction with a methanol:water mixture (80:20, *v/v*; 30 mL) at 25 ºC and 150 rpm during 1 h, followed by filtration through a Whatman filter paper No. 4. The residue was then extracted with one additional 30 mL portion of the hydromethanolic mixture. The combined extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and further lyophilized.

Phenolic compounds

The lyophilized extracts, infusions and decoctions were re-dissolved in methanol:water (80:20, *v/v*) and pure water, respectively, to determine the phenolic profiles by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA), as previously described (Guimarães et al., 2013). Double online detection was carried out with a diode array detector (DAD) using 280 nm and 370 nm as the preferred wavelengths connected in line with a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany). The phenolic compounds were identified by comparison of their retention times, UV-vis and mass spectra with those obtained from standard compounds, if existing. Otherwise, peaks were tentatively identified by comparing the obtained information with previous studies performed in our laboratory (Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, et al., 2015; Dias et al., 2016a) and available data reported in the literature. For quantitative analysis, individual standards calibration curves were constructed based on the area of the peaks recorded at 280 nm or 370 nm. For the identified phenolic compounds with no available commercial standard, the quantification was performed based on the calibration curve of a similar compound belonging to the same phenolic group. The results were expressed in mg per g of lyophilized extract or infusion and decoction.

Antioxidant activity

The lyophilized extracts, infusions and decoctions were re-dissolved in methanol:water (80:20, *v/v*) and water, respectively, to obtain stock solutions of 0.625 mg/mL, which were further diluted to obtain a range of concentrations for antioxidant activity evaluation. DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated through the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine brain homogenates was evaluated by the decrease in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Barros et al., 2013). The final results were expressed as EC_{50} values (μg/mL), sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control.

Statistical analysis

All the extractions were performed in triplicate and all the assays were also carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analysed using a Student´s t-test, in order to determine the significant difference between two different samples, with p = 0.05. In the case of being more then two samples the statistical analyses was performed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with p = 0.05 This treatment was carried out using SPSS v. 23.0 program.

4.1.3. Results and Discussion

Nutritional and chemical characterization of the lyophilized plant material and aqueous consumption forms

Data on the nutritional and chemical composition of the *in vitro* cultured vegetative parts of *F. vesca* are shown in **Table 39**. Carbohydrates (including fiber) were the most abundant macronutrient (84 g/100 g dw), followed by proteins, ash and fat (7, 6.5 and 2 g/100 g dw, respectively). Comparing to wild grown vegetative parts of *F. vesca* (Dias, Barros, Morales et al., 2015), the *in vitro* sample presented higher content of protein and lower content of ash, whereas the values of fat and carbohydrates are similar.

Fourteen different fatty acids were found, being more than half polyunsaturated fatty acids (PUFA), mainly linoleic acid (C18:2n6, 16%) and γ-linolenic acid (C18:3n6, 38%). Palmitic acid (C16:0) was also found in high levels (22%). Dias, Barros, Morales et al. (2015) reported lower percentages of total polyunsaturated fatty acids (41%) and higher levels of saturated fatty acids (53%) in wild grown vegetative parts of *F. vesca*. These results are motivating, since PUFA are components of membrane phospholipids, serve as precursors of some hormones with vital roles in the human body and are also important in the protection against some diseases such as rheumatoid arthritis, psoriasis and some age related diseases such as Alzheimer's (Patil & Gislerød, 2006).

The profile of soluble sugars in the lyophilized plant material (**Table 39**) and in the aqueous preparations (**Table 40**) was very similar, being glucose the most abundant in all samples (4 g/100 g dw in the dry sample, 9 mg/100 mL in the infusion and 10 mg/mL in the decoction preparation). Fructose was the second major sugar found in the lyophilized plant material and in the decoction (3 g/100 dw and 7 mg/100 mL, respectively), while for infusion sucrose (6 mg/100 mL) appeared as the second major sugar. Comparing with the results obtained by Dias, Barros, Morales, et al. (2015), the *in vitro* grown sample showed higher content of soluble sugars than the wild grown vegetative parts; furthermore, xylose was not previously described in the dry sample of *F. vesca*. These findings might indicate that the plant is producing larger amounts of sugars to maintain its vital functions of growth and development since it is limited by the *in vitro* culture itself. *In vitro* plants have and incipient photosynthesis, and because of that, have a large amount of sugars in the medium, but some plants, *in vitro* conditions, have an photosynthetic apparatus more developed than others, and if so, they can produced and store more sugars like glucose, mannose, xylose or even raffinose, the type of sugar depends of the type of transportation in phloem, and that depends of the genetic characteristics of the plant species.

Table 39. Nutritional value, fatty acids, soluble sugars, organic acids and tocopherols content of *in vitro* cultured vegetative parts from wild *Fragaria vesca* L. (mean ± SD).

Nutritional value	**(g/100 g dw)**	**Soluble sugars**	**g/100 g dw**
Fat	2.37 ± 0.01	Xylose	0.98 ± 0.02
Proteins	7.27 ± 0.12	Fructose	2.55 ± 0.17
Ash	6.53 ± 0.20	Glucose	3.94 ± 0.17
Total carbohydrates	83.83 ± 0.06	Sucrose	2.20 ± 0.01
Energy (kcal/100 g dw)	385.73 ± 0.57	Trehalose	0.35 ± 0.06
		Sum	10.04 ± 0.26
Fatty acids	**(relative percentage)**	**Organic acids**	**g/100 g dw**
C6:0	0.16 ± 0.01	Oxalic acid	3.76 ± 0.06
C8:0	0.34 ± 0.01	Quinic acid	0.85 ± 0.05
C10:0	0.22 ± 0.02	Shikimic acid	0.002 ± 0.001
C12:0	2.65 ± 0.12	Ascorbic acid	0.02 ± 0.01
C14:1	3.03 ± 0.07	Succinic acid	1.58 ± 0.20
C15:1	0.61 ± 0.04	Fumaric acid	tr
C16:0	21.37 ± 0.17	Sum	6.20 ± 0.21
C16:1	0.56 ± 0.09		
C17:0	0.57 ± 0.004		
C18:1n9	5.62 ± 0.08		
C18:2n6	16.11 ± 0.05		
C18:3n6	37.54 ± 0.46	**Tocopherols**	**mg/100 g dw**
C20:1	6.85 ± 0.05	α-Tocopherol	98.54 ± 0.90
C22:1n9	4.38 ± 0.01	β-Tocopherol	4.90 ± 0.04
SFA	**25.01 ± 0.15**	γ-Tocopherol	24.86 ± 0.23
MUFA	**21.34 ± 0.56**	δ-Tocopherol	11.04 ± 0.10
PUFA	**53.56 ± 0.41**	Sum	139.35 ± 1.27

nd- not detected; tr- traces. SFA- saturated fatty acids, MUFA- monounsaturated fatty acids, PUFA- polyunsaturated fatty acids. Calibration curves for organic acids: oxalic acid (y =9x106 x + 377946, R^2=0.994); quinic acid (y =6010607 x + 46061, R^2=0.9995); shikimic acid (y =7x107 x + 175156, R^2=0.9999); ascorbic acid (y =108 x + 751815, R^2=0.998); succinic acid (y =603298 x + 4994.1, R^2=1) and fumaric acid (y =154862 x + 1x106, R^2=0.9977). (<LOD: 12.6, 24, 6, 3, 19 and 0.080 µg/mL for oxalic, quinic, shikimic, ascorbic, succinic and fumaric acid respectively); (<LOQ: 42, 81, 19, 11, 64 and 0.26 µg/mL for oxalic, quinic, shikimic, ascorbic, succinic and fumaric acid respectively).

Regarding organic acids, oxalic acid was the majority one found in the lyophilized plant material (4 g/100 dw) followed by succinic acid (6 g/100 dw); other acids, and among them ascorbic acid, were found in very low levels. Oxalic acid was also the predominant acid found in the infusions, although in that case followed by quinic acid (6 and 5 mg/100 mL, respectively), and quite similar amounts of these two organic acids were found in decoctions. As for sugars and fatty acids, the organic acids content in the *in vitro* cultured samples was significantly higher than the one reported by Dias, Barros, Morales, et al. (2015) in wild grown vegetative parts and corresponding infusions and decoctions.

The four tocopherol isoforms were found in the lyophilized plant material, with α-tocopherol as predominant (99 mg/100 dw) followed by γ-tocopherol (25 mg/100 dw). However, only α- and β-tocopherol were detected in the infusions and decoctions, being the latter the majority one in both preparations. The lower content of tocopherols in the aqueous preparations was expected due to their lipophilic character. Quite interestingly, the

lyophilized plant material, infusions and decoctions of the *in vitro* cultured samples herein studied showed much higher tocopherol levels (139 mg/100 dw, 1.98 and 1.66 μg/100 mL, respectively) than the equivalent ones obtained from wild grown vegetative parts of *F. vesca* (7 mg/100 dw, 0.19 and 0.22 μg/100 mL, respectively), in which only one isoform (α-tocopherol) was reported in the infusions and decoctions (Dias, Barros, Morales, et al., 2015).

Table 40. Soluble sugars, organic acids and tocopherols contents in infusions and decoctions prepared from in vitro cultured vegetative parts of wild Fragaria vesca L. (mean ± SD).

	Infusions	**Decoctions**	***t-Student p-value***
Soluble sugars	**mg/100 mL**	**mg/100 mL**	
Xylose	2.85 ± 0.07	2.89 ± 0.20	0.572
Fructose	6.12 ± 0.15	7.15 ± 0.39	<0.001
Glucose	9.49 ± 0.05	10.14 ± 0.80	0.013
Sucrose	6.48 ± 0.27	3.29 ± 0.20	<0.001
Trehalose	1.17 ± 0.14	0.66 ± 0.14	<0.001
Sum	26.13 ± 0.23	24.13 ± 1.46	<0.001
Organic acids	**mg/100 mL**	**mg/100 mL**	
Oxalic acid	6.44 ± 0.01	5.55 ± 0.01	<0.001
Quinic acid	4.958 ± 0.003	5.572 ± 0.001	<0.001
Shikimic acid	0.086 ± 0.001	0.117 ± 0.001	<0.001
Fumaric acid	tr	tr	-
Sum	11.48 ± 0.26	11.24 ± 0.24	<0.001
Tocopherols	**μg/100 mL**	**μg/100 mL**	
α-Tocopherol	0.16 ± 0.02	0.17 ± 0.01	0.310
β-Tocopherol	1.82 ± 0.08	1.49 ± 0.01	<0.001
Sum	1.98 ± 0.06	1.66 ± 0.01	<0.001
	Infusions	**Decoctions**	***t-Student p-value***

tr- traces. Calibration curves for organic acids: oxalic acid (y =9x106 x + 377946, R^2=0.994); quinic acid (y =6010607 x + 46061, R^2=0.9995); shikimic acid (y =7x107 x + 175156, R^2=0.9999); ascorbic acid (y =108 x + 751815, R^2=0.998); succinic acid (y =603298 x + 4994.1, R^2=1) and fumaric acid (y =154862 x + 1x106, R^2=0.9977).(<LOD: 12.6, 24, 6 and 0.080 μg/mL for oxalic, quinic, shikimic and fumaric acid respectively); (<LOQ: 42, 81, 19 and 0.26 μg/mL for oxalic, quinic, shikimic and fumaric acid respectively).

Phenolic profile and antioxidant activity of the hydromethanolic extracts and aqueous consumption forms

Table 41presents the peak characteristics (retention time, wavelength of maximum absorption and mass spectral data), tentative identification and quantification of the phenolic compounds present in the hydromethanolic extracts, infusions and decoctions of the *in vitro* cultured vegetative parts of *F. vesca.* An exemplificative phenolic profile of the hydromethanolic extract recorded at 280 and 370 nm is shown in **Figure 21**. Thirty different phenolic compounds where identified in the samples, four phenolic acids (peaks 8, 11, 12 and 14), twelve ellagic acid derivatives (peaks 1, 3, 9, 10, 15, 17, 18, 24, 25, 28, 29 and 30), four flavan-3-ols (peaks 2, 4, 6, and 7), nine flavonols (peaks 5, 13, 16, 19, 20, 21, 23, 26 and 27) and one dihydroflavonol (peak 22). The hydromethanolic extracts and the aqueous

preparations showed a very similar profile, only distinguished at the quantification level and for the absence of some compounds in infusions and decoctions.

Most of the detected compounds (i.e., peaks 1-6, 8-10, 13, 15, 17-30) have been previously described in wild *F. vesca* and other *Fragaria* species (Del Bubba et al., 2012; Dias et al., 2016a; Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, et al., 2015; Gasperotti et al., 2013; Simirgiotis & Schmeda-Hirschmann, 2010; Sun et al., 2014), so that their identities are assumed herein. To the author's best knowledge, peaks 7, 11, 12, 14 and 16 have not been reported before in *F. vesca*. Although no information could be obtained regarding mass characteristics of peak 7, it was tentatively associated to a flavan-3-ol based on the characteristic shape of its UV spectrum; the observed λ_{max} at 272 nm would point to a gallocatechin or a derived proanthocyanidin (e.g., a prodelphinidin), expected to have maximum wavelength at lower values than catechins and related procyanidins (278-280 nm). Peaks 11 and 12 were tentatively identified as coumaroylquinic acid isomers according to their pseudomolecular ion $[M-H]^-$ *m/z* at 337, releasing fragments at *m/z* 191 and *m/z* 163 corresponding to the deprotonated quinic acid and the coumaric acid moiety, respectively. Peak 14 was identified as feruloylquinic acid based on its pseudomolecular ion $[M-H]^-$ *m/z* at 367 and the production of a major daughter ion at *m/z* 193 [ferulic acid-H]$^-$. Peak 16 showed a UV spectrum with λ_{max} at 368 nm, a pseudomolecular ion $[M-H]^-$ *m/z* at 477 and MS^2 fragments at *m/z* 315 and 301, which allowed its tentative identification as isorhamnetin-*O*-glucoside.

The methanolic extract presented higher concentrations of total phenolic compounds (44 mg/g) than the aqueous preparations (26-31 mg/g), mainly due to its greater content of ellagic acid derivatives (19 mg/g). Peak 17 (sanguiin h10 isomer) was the majority compound found in the methanolic extracts, followed by peak 4 (procyanidin dimer). Different observations regarding the phenolic profile of *in vitro* grown leaves of *F. vesca* were made by Yildirim & Turker (2014), who only reported two common compounds with those detected in our study (i.e., (+)-catechin and a procyanidin dimer), and in much lower amounts.

Smaller contents of phenolic compounds were determined in the present study than previously found in wild grown vegetative parts (Dias, Barros, Fernandes, et al., 2015). A possible explanation might be the short stationary phase in the growth of the *in vitro* cultured plants, which would lead to lower yields in the production of secondary metabolites, due to the inhibition of the action of enzymes normally present in mature plants (Dias et al., 2016). Furthermore, *in vitro* grown plants are not as subjected to environmental stress as wild plants, a factor that is known to influence phenolic accumulation. All in all, this could mean that *in vitro* grown *F. vesca* would need to be elicited to produce higher amounts of phenolics.

Table 41 Retention time (Rt), wavelengths of maximum absorption (λmax), mass spectral data, tentative identification and quantification of phenolic compounds in hydromethanolic extracts, infusions and decoctions of the *in vitro* cultured vegetative parts of wild *Fragaria vesca* L.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Reference used for identification	Extracts	Infusions	Decoctions
1	4.7	258	783	481(3),301(30)	Bis-HHDP-hexoside[B]	(Dias, Barros, Fernandes, et al., 2015; M. I. Dias et al., 2016a; Gasperotti et al., 2013; Simirgiotis & Schmeda-Hirschmann, 2010; Sun et al., 2014)	1.94 ± 0.08[a]	1.27 ± 0.02[c]	1.34 ± 0.13[b]
2	5.6	278	451	289(100)	(Epi)catechin hexoside[A]	(Del Bubba et al., 2012; Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, et al., 2015)	4.30 ± 0.06[b]	3.78 ± 0.02[c]	8.24 ± 0.06[a]
3	6.05	256	783	481(25),301(14)	Bis-HHDP-hexoside[B]	(Dias, Barros, Fernandes, et al., 2015; Simirgiotis & Schmeda-Hirschmann, 2010; Sun et al., 2014)	0.46 ± 0.18[c]	0.72 ± 0.14[b]	1.58 ± 0.08[a]
4	6.87	278	577	451(33), 425(65), 407(100), 289(75), 287(17)	Procyanidin dimer[A]	(Del Bubba et al., 2012; Dias et al., 2016a; M. I. Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, et al., 2015; Sun et al., 2014; Yildirim & Turker, 2014)	6.13 ± 0.02[a]	3.31 ± 0.08[b]	3.24 ± 0.42[b]
5	7.19	356	639	463(69),301(59)	Quercetin glucuronyl-hexoside[E]	(Dias, Barros, Fernandes, et al., 2015)	0.08 ± 0.01[c]	0.14 ± 0.01[b]	0.21 ± 0.01[a]
6	7.76	278	289	245(35), 203(32), 137(32)	(+)-Catechin[A]	(Del Bubba et al., 2012; Dias et al., 2016a; Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, et al., 2015; Simirgiotis & Schmeda-Hirschmann, 2010; Yildirim & Turker, 2014)	3.69 ± 0.15[c]	4.77 ± 0.01[b]	5.57 ± 0.021[a]

7	8.35	272	-	425(65), 407(), 289(100)	Gallocatechin-related flavan-3-ol[A]		2.80 ± 0.10[a]	1.53 ± 0.03[c]	2.41 ± 0.01[b]
8	9.63	332	355	193(18),175(100),161(20)	Ferulic acid hexoside[C]	(Sun et al., 2014)	0.42 ± 0.01[a]	0.24 ± 0.02[c]	0.27 ± 0.01[b]
9	14.5	270	935	633(25),301(21)	Galloyl-bis-HHDP-glucose isomer[B]	(Del Bubba et al., 2012; Dias et al., 2016a; Gasperotti et al., 2013; Simirgiotis & Schmeda-Hirschmann, 2010; Sun et al., 2014)	2.25 ± 0.03	nd	nd
10	15	372	463	301(100)	Ellagic acid hexoside[B]	(Dias et al., 2016a)	0.44 ± 0.01[a]	0.33 ± 0.03[b]	0.22 ± 0.01[c]
11	15.45	316	337	191(7),173(35),163(10),155(5)	Coumaroylquinic acid isomer[F]		0.56 ± 0.02a	0.47 ± 0.06[b]	0.47 ± 0.01[b]
12	15.7	316	337	191(8),173(38),163(12),155(6)	Coumaroylquinic acid isomer[F]		0.21 ± 0.01[b]	0.41 ± 0.10[a]	0.24 ± 0.01[b]
13	16.61	352	623	301(100)	Quercetin rhamnosyl-glucuronide[E]	(Dias, Barros, Fernandes, et al., 2015)	0.20 ± 0.01[b]	0.21 ± 0.01[b]	0.23 ± 0.01[a]
14	16.75	320	367	193(100),191(16),173(14),149(25)	Feruloylquinic acid[C]		0.20 ± 0.01[c]	0.38 ± 0.01[b]	0.32 ± 0.01[a]
15	17.07	372	433	301(100)	Ellagic acid pentoside[B]	(Del Bubba et al., 2012; Dias et al., 2016a; Simirgiotis & Schmeda-Hirschmann, 2010; Sun et al., 2014)	0.31 ± 0.01	tr	nd
16	17.59	368	477	315(35),301(100)	Isorhamnetin-O-glucoside[D]		1.18 ± 0.03[a]	0.97 ± 0.01[b]	0.79 ± 0.01[c]
17	17.93	262	1567	935(95), 783(5),631(2),613(13), 301(6)	Sanguiin h10 isomer[B]	(Dias et al., 2016a; Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, et al., 2015; Gasperotti et al., 2013)	10.48 ± 0.13	nd	nd
18	19.29	250/sh370	447	301(100)	Ellagic acid rhamnoside[B]	(Del Bubba et al., 2012; Dias et al., 2016a; Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, et al., 2015; Gasperotti et al., 2013; Simirgiotis & Schmeda-Hirschmann, 2010; Sun et al., 2014)	0.31 ± 0.01[b]	0.51 ± 0.04[a]	0.29 ± 0.01[b]
19	19.3	346	607	285(100)	Kaempferol rhamnosyl-glucuronide[H]	(Dias, Barros, Fernandes, et al., 2015)	0.71 ± 0.01[b]	0.70 ± 0.02[b]	0.89 ± 0.01[a]
20	19.87	356	477	301(100)	Quercetin glucuronide[E]	(Del Bubba et al., 2012; Dias, Barros, Fernandes, et al., 2015; Simirgiotis & Schmeda-Hirschmann, 2010; Sun et al., 2014)	0.53 ± 0.01[a]	0.46 ± 0.01[c]	0.49 ± 0.01[b]

21	20.04354	637	315(95),300(26)	Methylquercetin rhamnosyl glucuronide[E]	(Dias, Barros, Fernandes, et al., 2015)	0.22 ± 0.01[c]	0.23 ± 0.01[b]	0.27 ± 0.01[a]
22	20.18292/sh338435		303(100)	Taxifolin-pentoside[G]	(Dias, Barros, Oliveira, et al., 2015; Sun et al., 2014)	2.81 ± 0.02[a]	2.23 ± 0.08[b]	1.67 ± 0.19[c]
23	20.56356	463	301(100)	Quercetin 3-O-glucoside[E]	(Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, et al., 2015; Simirgiotis & Schmeda-Hirschmann, 2010; Sun et al., 2014)	0.24 ± 0.01	nd	nd
24	21.11254/sh370301		284(4),256(3),229(4), 185(4)	Ellagic acid[B]	(Del Bubba et al., 2012; Dias et al., 2016a; Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, et al., 2015; Simirgiotis & Schmeda-Hirschmann, 2010; Sun et al., 2014)	0.89 ± 0.04[b]	1.73 ± 0.01[a]	tr
25	23.35378	447	315(28),300(100)	Methyl ellagic acid pentoside[B]	(Del Bubba et al., 2012; Dias et al., 2016a; Sun et al., 2014)	tr	tr	1.64 ± 0.09
26	23.9 348	461	285(100)	Kaempferol-glucuronide[H]	(Simirgiotis & Schmeda-Hirschmann, 2010)	0.30 ± 0.01[a]	0.26 ± 0.02[b]	0.29 ± 0.03[a]
27	24.21348	447	285(100)	Kaempferol-hexoside[H]		0.13 ± 0.01[a]	0.09 ± 0.01[c]	0.10 ± 0.01[b]
28	24.83364	447	315(12),300(100)	Methyl ellagic acid pentoside[B]	(Del Bubba et al., 2012; Dias et al., 2016a; Sun et al., 2014)	tr	tr	tr
29	25.26248/sh372461		315(89),301(38)	Dimethyl ellagic acid pentoside[B]	(Del Bubba et al., 2012; Dias et al., 2016a; Dias, Barros, Oliveira, et al., 2015; Gasperotti et al., 2013; Sun et al., 2014)	1.76 ± 0.05[a]	0.98 ± 0.03[b]	0.52 ± 0.03[c]
30	26.98368	461	315(37),301(100)	Dymethyl ellagic acid pentoside[B]	(Del Bubba et al., 2012; Dias et al., 2016a; Dias, Barros, Fernandes, et al., 2015; Dias, Barros, Oliveira, et al., 2015; Gasperotti et al., 2013; Sun et al., 2014)	tr	tr	tr
				Total phenolic acids		1.39 ± 0.01[b]	1.49 ± 0.04[a]	1.29 ± 0.04[c]
				Total ellagic acid derivatives		18.85± 0.045[a]	5.54 ± 0.07[b]	5.60 ± 0.03[b]
				Total flavan 3-ols		6.41 ± 0.01[a]	5.28 ± 0.09[b]	4.93 ± 0.28[c]

	Total flavonols	16.91 ± 0.22[b]	13.39 ± 0.10[c] 19.47 ± 0.16[a]
	Total phenolic compounds	43.55 ± 0.25[a]	25.70 ± 0.16[c] 31.29 ± 0.05[b]

Different letters mean significant statistical differences between samples ($p<0.05$), where "a" and "c" correspond to the highest and lowest values, respectively. tr-traces; nd- not detected. Standard calibration curves: (A) catechin (y =158.42 x +11.38, R^2=0.999); (B) ellagic acid (y = 32.748 x + 77.8, R^2=0.9994); (C) ferulic acid (y =525.36 x +233.82, R^2=0.9994); (D) isorhametin-3-*O*-glucoside (y =218.26 x -0.98, R^2=1); (E) quercetin-3-*O*-glucoside (y = 253.52 x -11.615, R^2=0.9984); (F) *p*-coumaric acid (y =706.09 x +1228.1, R^2=0.9989); (G) taxifolin (y =224.31 x +148.41, R^2=0.999); (H) kaempferol-3-*O*-glucoside (y = 288.55 x -4.0503, R^2=1).

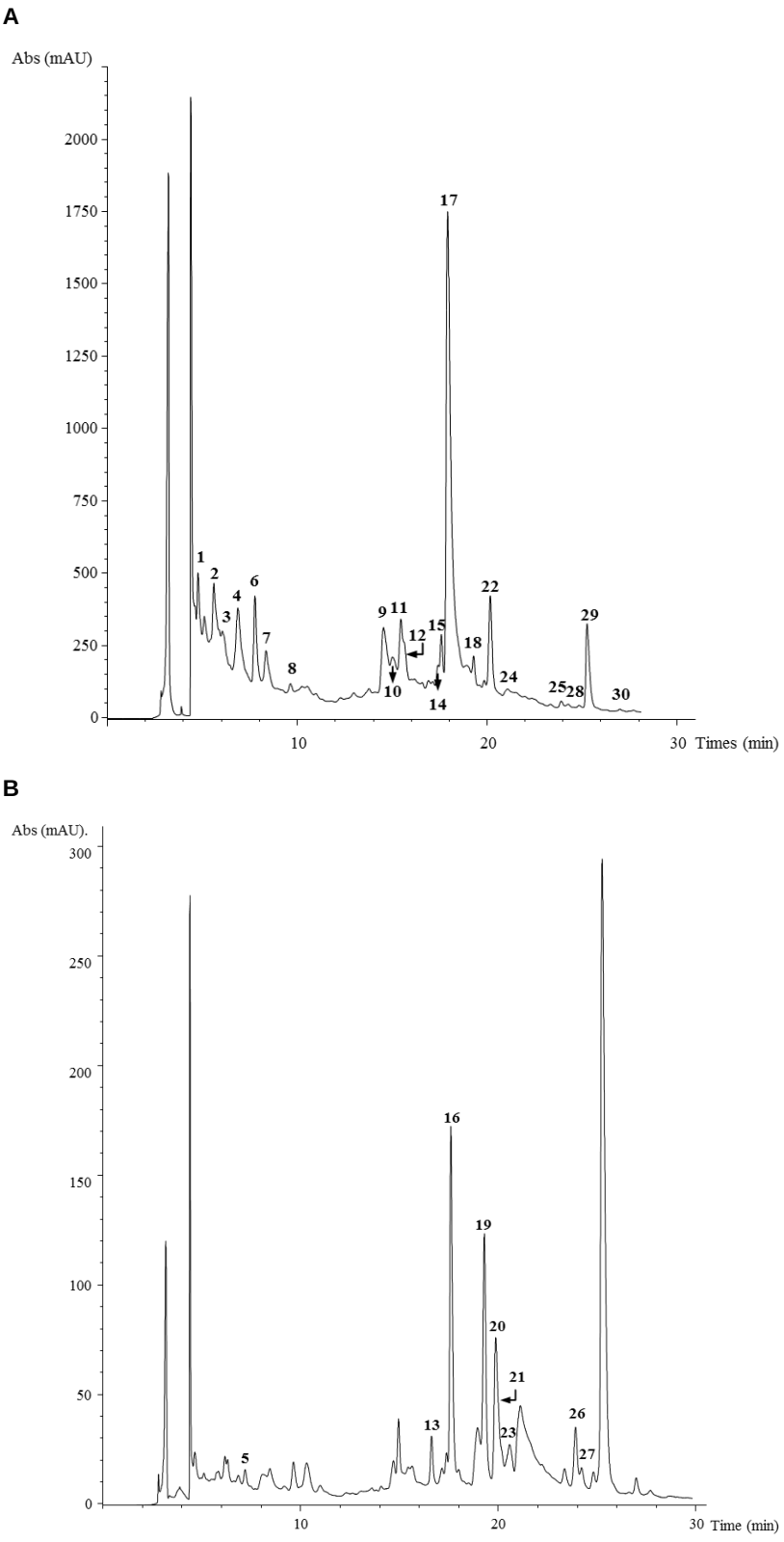

Figure 21. HPLC chromatograms recorded at 280 nm (A) and 370 nm (B) showing the phenolic profile of the hydromethanolic extract of the *in vitro* cultured *Fragaria vesca* L

Antioxidant activity of the hydromethanolic extracts and aqueous consumption forms

The results on the antioxidant activity of the hydromethanolic extract, infusions and decoctions of *in vitro* cultured vegetative parts are collected in**Table 42**. The hydromethanolic extract showed the highest DPPH scavenging activity and reducing power (EC_{50}= 83 and 57 μg/mL, respectively), while for β-carotene bleaching inhibition and TBARS inhibition the lowest EC_{50} values were observed for the infusions (EC_{50}= 52 and 25 μg/mL, respectively). The results found for reducing power can be moderately correlated with the contents of phenolic acid derivatives in the samples (r^2=0.777), while for TBARS inhibition the results were highly correlated with these compounds (r^2=0.903), but especially with ellagic acid derivatives (r^2=0.9908), as well as with flavonols (r^2=0.9152).

The antioxidant activity found for the hydromethanolic extract in the DDPH scavenging, reducing power and β-carotene assays was higher than the one observed for the extracts of wild grown vegetative parts of *F. vesca* L., despite these latter contained higher concentrations of phenolic compounds (Dias, Barros, Fernandes, et al., 2015). This could be due to the different phenolic profiles existing in both types of samples, but also to the presence of other components in the extracts, such as sugars, organic acids or tocopherols, which occur in higher levels in the *in vitro* cultured sample, and that also have an influence on the antioxidant potential.

Table 42. Antioxidant activity of the hydromethanolic extracts, infusions and decoctions of *in vitro* cultured vegetative parts of wild *Fragaria vesca* L.

EC_{50} values (μg/mL)			
DPPH scavenging activity	82.5 ± 3.1^{b}	86.9 ± 0.9^{ab}	93.6 ± 10.1^{a}
Reducing power	57.0 ± 0.1^{c}	75.9 ± 0.4^{a}	62.0 ± 0.3^{b}
β-carotene bleaching inhibition	54.4 ± 1.9^{a}	52.4 ± 1.0^{b}	54.2 ± 0.1^{a}
TBARS inhibition	230.3 ± 16.1^{a}	25.3 ± 0.8^{b}	27.1 ± 1.6^{b}

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Different letters mean significant statistical differences between samples ($p<0.05$), where “a” and “c” correspond to the highest and lowest values, respectively.

Overall, the plant tissue culture technique applied to *Fragaria vesca* L. proved to be a suitable approach to obtain higher contents of proteins, polyunsaturated fatty acids, soluble sugars, organic acids (including ascorbic acid) and tocopherols (mainly α-tocopherol). Furthermore, the hydromethanolic extracts of the *in vitro* grown samples showed greater antioxidant activity than the ones obtained from wild grown *F. vesca*. In contrast, although the phenolic profile was similar to that observed in wild grown plants, lower levels of total phenolic compounds were accumulated in the *in vitro* cultured samples. Further studies

should be required so as to check whether this limitation might be overcome by elicitation of plant growth.

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5.

Microencapsulação de extratos bioativos de *Fragaria vesca* L. e incorporação numa matriz alimentar

Neste capítulo apresenta-se a microencapsulação como ferramenta para a proteção de bioativos. Apresenta-se o perfil fenólico individual e a atividade antioxidante de extratos aquosos e metanol: água de *Fragaria vesca* L. silvestre e comercial, e descreve-se o desenvolvimento de um produto alimentar (gelatina de *k*-carregenina) enriquecido com microesferas de alginato contendo o extrato mais bioativo (infusão da amostra silvestre).

5.1. Formulação bioativa baseada nas partes vegetativas de *Fragaria vesca* L.: caraterização química e aplicação em gelatina de *k*-carragenina

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A bioactive formulation based on *Fragaria vesca* L. vegetative parts: chemical characterization and application in k-carrageenan gelatin.

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Abstract

A nutraceutical formulation based on the vegetative parts of the wild strawberry, *Fragaria vesca* L., was developed by using a microencapsulated extract (lyophilized infusion form). For that purpose, a process based on an atomization/coagulation technique with alginate as the wall material was applied. Among the tested hydromethanolic and aqueous extracts, both obtained from wild and commercial samples, the infusion of a wild species

emerged as the most antioxidant one. The higher amounts of flavonols and flavan-3-ols found in the aqueous extracts seem to be responsible for this greater antioxidant activity. Furthermore, the developed nutraceutical formulation was applied in *k*-carrageenan gelatin, being observed that the antioxidant properties of the extract were preserved, as compared with the free form. In conclusion the antioxidant activity of the *Fragaria vesca* L. vegetative parts was demonstrated, as well as, the advantages of using microencapsulation to produce effective nutraceutical formulations.

Keywords: Fragaria vesca L.; Vegetative parts; Hydromethanolic/Aqueous extracts; Microencapsulation; Alginate; *k*-Carrageen

5.1.1. Introduction

Wild strawberry, *Fragaria vesca* L., is a herbaceous perennial plant from the Rosaceae family. It is widely spread across Europe, North America and Canada, and it can be found in roadsides and slopes, as also in forests (Castroviejo et al., 1998). The antioxidant properties of *F. vesca* fruits and leaves (Raudonis, Raudone, Jakstas & Janulis 2012; Nuñez-Mancilla, Pérez-Won, Uribe, Vega-Gálvez & Scala 2013; Žugić et al., 2014), pulp (Özşen & Erge, 2013), achenes, thalamus (Cheel, Theoduloz, Rodríguez, Caligari & Schmeda-Hirschmann 2007) and roots (Dias, Barros, Oliveira, Santos-Buelga & Ferreira 2015a) have been described. Although being mostly known by the sweat small fruits, their vegetative parts are also consumed as decoctions for hypertension treatment and due their detoxifying, diuretic, stimulant and dermatological protective properties (Neves, Matos, Moutinho, Queiroz & Gomes 2009; Camejo-Rodrigues, Ascensão, Bonet & Vallès, 2012).

The bioactive properties of different strawberry parts (fruits, leaves and roots) have been related with the presence of various phenolic compounds, such as hydroxycinnamic and ellagic acids derivatives (e.g., ellagitannins), and flavonols (Clifford & Scalbert, 2000; Zheng, Wang, Wang & Zheng 2007; Pinto, Lajolo & Genovese 2008; Simirgiotis & Schmeda-Hirschmann, 2010; Bubba, Checchini, Chiuminatto, Doumett, Fibbi & Giordani 2012; Gasperotti et al., 2013; Dias et al., 2014; Sun, Liu, Yang, Slovin & Chen 2014). The presence of these bioactive compounds makes this plant very appealing, not only for consumers, but also for food and pharmaceutical industries. However, after ingestion, phenolic compounds can suffer transformations to methylate, glucuronate and sulphate metabolites (Heleno, Martins, Queiroz & Ferreira, 2015). In fact, the stability and functionality of this type of compounds within the human body, and consequently their bioavailability, is highly influenced by the ingested amount, structure and chemical form, molecular interactions and the organism itself (Holst & Williamson, 2008; Leong & Oey, 2012). A major problem of

phenolic compounds is the poor solubility in water and the low permeability due the absence of specific receptors at the small intestinal epithelial cells surface (Li, Jiang, Xu & Gu, 2015).

To overcome these problems microencapsulation emerges as a reliable response to protect and stabilize bioactive compounds/extracts, also offering a controlled or targeted delivery (Dias, Ferreira & Barreiro, 2015b). The microcapsules can present sizes ranging from 1 to 1000 micrometers and two main types of morphology: reservoir and matrix type. In the first case a wall/shell protects a core (bioactive) and in the second one the bioactive is dispersed along a continuous polymeric matrix. The controlled release of the bioactives, that should be tailored according to the final application of the microencapsulated product, can be achieved by several mechanisms, for example, mechanical action, heat gradients, diffusion, pH modification, biodegradation and dissolution. Water-soluble polymers are the most used wall materials (Dias et al., 2015b), being alginate the most common one; their physiochemical properties have been intensively studied proving to have good stability, biocompatibility, exudate-retaining ability and some antimicrobial activity (Goh, Heng & Chan, 2012).

Microencapsulation technique could find many applications in fields such as the pharmaceutical, food, agriculture, biomedical and even electronics (Martins, Barreiro, Coelho & Rodrigues, 2014a; Martins et al., 2014b). As far as we know there are no studies using *Fragaria* species, namely in what concerts the microencapsulation of *F. vesca* extracts and their subsequent use to enrich food matrices such as *k*-carrageenan gelatin.

k-Carrageenan is a linear anionic heteropolyshaccharide extracted from red algae and composed by galactose and anhydrogalactose units containing ester sulfate groups, (Baeza, Carp, Pérez & Pilosof, 2002). It is widely used in the food industry as gelling, stabilizing and thickening agents. The gelling process occurs upon solution cooling, being affected by factors such as salt concentration, temperature, and pH, forming generally very firm gels (Bartkowiak & Hunkeler, 2001; Grenha et al., 2010).

In the present study, *F. vesca* vegetative parts (wild and commercial samples) were used to obtain hydromethanolic and aqueous extracts. After evaluation of their antioxidant activity and establishment of the individual phenolic profile, the most active extract was protected by microencapsulation through the atomization/coagulation technique using alginate as the wall material. An applicability assay was developed using *k*-carrageenan gelatin as food matrix, as a way to explore new nutraceutical formulations for food applications.

5.1.2. Materials and methods

Samples

The commercial samples of *Fragaria vesca* L. vegetative parts (leaves and stems) were purchased in a local supermarket. The wild vegetative parts of *F. vesca* were collected in Serra da Nogueira, Bragança, North-eastern Portugal, in July 2013. Morphological key characters from the Flora Iberica (Castroviejo et al., 1998) were used for plant identification. Voucher specimens (nº 9687) are deposited in the School of Agriculture Herbarium (BRESA). All the samples were lyophilized (FreeZone 4.5, Labconco, Kansas, USA) and powdered (20 mesh).

Standards and Reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic acid was purchased from Prolabo (WWR International, France). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was acquired from Sigma (St. Louis, MO, USA. Phenolic standards were from Extrasynthèse (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Sodium alginate was obtained from Fluka Chemie (Steinheim, Switzerland) and calcium chloride 2-hydrate was purchased from Panreac (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Preparation of the hydromethanolic and aqueous extracts

Hydromethanolic extraction was performed by stirring the powdered sample (1 g) with 30 mL of a methanol:water mixture (80:20, *v/v*) at 25 ºC and 150 rpm during 1 h, followed by filtration through a Whatman filter paper No. 4. The residue was then extracted with one additional 30 mL portion of the hydromethanolic mixture. For each sample, the combined extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and further lyophilized.

For infusions preparation, each sample (1 g) was added to 200 mL of boiling distilled water (pH 6.6) at 100 ºC, left to stand at room temperature for 5 min, and then filtered under reduced pressure (0.22 µm, through Whatman No. 4 paper).

For decoctions preparation, each sample (1 g) was added to 200 mL of distilled water (pH 6.6), heated (heating plate, VELP scientific, Keyland Court, NY, USA) and le to boil during 5 min at 100 ^{o}C, in a closed recipient to prevent evaporation. The mixture was left to stand for 5 min and then filtered under reduced pressure (0.22 µm, through Whatman No. 4 paper). The obtained infusions and decoctions were frozen and lyophilized.

Phenolic compounds analysis

The lyophilized extracts were re-dissolved in a water:methanol mixture (80:20, *v/v*) or in pure water to determine the phenolic profiles by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA), as previously described elsewhere (Barros et al., 2013). Double online detection was carried out with diode array detector (DAD) using 280 nm and 370 nm as the preferred wavelengths in line with a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany). The phenolic compounds were identified by comparing their retention times, UV-vis and mass spectrum with those obtained from standard compounds, if existing. Otherwise, peaks were tentatively identified by comparing the obtained information with available data reported in the literature. For quantitative analysis, individual standards calibration curves were constructed based on the UV signal: catechin (y=158.42x+11.38, R^2=0.999); ellagic acid (y=32.748x+77.8, R^2=0.999); gallic acid (y=421.11x+546.14, R^2=0.996); quercetin-3-*O*-glucoside (y=253.52x-11.615, R^2=0.999); quercetin-3-*O*-rutinoside (y=281.98x-0.3459, R^2=1); kaempherol-3-*O*-glucoside (y=288.55x-4.0503, R^2=1); kaempferol-3-*O*-rutinoside (y=239.16x-10.587, R^2=1) and *p*-coumaric acid (y=884.6x+184.49, R^2=0.999). For the identified phenolic compounds with no available commercial standard, the quantification was performed based on the calibration curve of a similar compound belonging to the same phenolic group. The results were expressed in mg per g of extract.

Antioxidant activity evaluation

The lyophilized extracts were re-dissolved in the methanol:water (80:20, *v/v*) or water to obtain stock solutions of 2.5 mg/mL, which were further diluted to obtain a range of concentrations for antioxidant activity evaluation.

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated through the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated

using the following formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Barros et al., 2013; Dias et al., 2015a). The final results were expressed as EC_{50} values (μg/mL), sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control.

Encapsulation of the most antioxidant extracts

Microspheres containing the lyophilized infusion of wild vegetative parts of *F. vesca*, were prepared by using an atomization/coagulation technique as previously described by the authors (Martins et al., 2014b). Briefly, sodium alginate was used as the matrix material and calcium chloride ($CaCl_2$) as the coagulation agent. The atomizing solution was prepared by firstly dissolve 50 mg of the lyophilized extract in 10 mL of distilled water under stirring followed by filtration to remove eventual non-soluble trace residues. Thereafter 400 mg of sodium alginate were added and the solution kept under stirring until complete dissolution was achieved. The obtained alginate solution containing the extract was then atomized using a NISCO Var J30 system (Zurich, Switzerland) at a feed rate of 0.3 mL/min and a nitrogen pressure of 0.1 bar. The generated microspheres were immediately coagulated by contacting with the $CaCl_2$ aqueous solution (250 mL at a concentration of 4%, *w/v*), for a period of 4 hours. The resulting microspheres were collected by filtration under reduced pressure, washed twice with distilled water, and further lyophilized and stored under dark conditions at 4 ^{o}C.

Microspheres were analysed by optical microscopy (OM) using a Nikon Eclipse 50i microscope (Tokyo, Japan) equipped with a Nikon Digital Sight camera and NIS Elements software for data acquisition and by SEM using a Phenom ProX desktop microscope (Eindhoven, The Netherlands). OM analysis was applied to assess the size and morphology of the microspheres after the atomization and coagulation stages, as well as after rehydration. SEM analysis was used to inspect final morphology of the lyophilized samples. The effective extract incorporation into the alginate matrix was investigated by FTIR analysis. For that purpose, spectrum of pure alginate, free extract of *F. vesca* and the corresponding microspheres were collected on a FTIR Bomen (model MB 104) by preparing KBr pellets at a sample concentration of 1% (w/w). Spectrum were recorded at a resolution of 4 cm^{-1} between 650 and 4000 cm^{-1} by co-adding 48 scans. The dry residue (DR) was calculated as the ratio between the dry (lyophilized) form and the corresponding wet microsphere weight (%, *w/w*). The evaluation of the encapsulation efficiency (EE) was performed through the quantification of the non-encapsulated extract. The encapsulation efficiency was calculated according to the following expression:

$$EE = [(M_{e\text{-}t} - M_{e\text{-}ne})/(M_{e\text{-}t})] \times 100$$

in which M_{e-t} represents the theoretical amount of extract, i.e. the amount of extract used in the microencapsulation process. M_{e-ne} corresponds to the non-encapsulated extract remaining after the encapsulation process. Since the extract corresponds to a complex mixture of several components, the major compound (quercetin *O*-glucuronide) was selected for EE evaluation. The quercetin *O*-glucuronide quantification was performed by HPLC based on the analysis of the coagulation and first washing solutions since in the second washing solution no extract components were detected.

Incorporation of free and microencapsulated F. vesca extracts in k-carrageenan gelatin

For the incorporation assay, the chosen food matrix was the most common gelling agent found in commercial gelatine, *k*-carrageenan. This strategy of using the gelling agent instead of a commercial gelatin was chosen to avoid the presence of additional antioxidant compounds, e.g ascorbic acid, typical of these formulations, which could mask the results.

The protocol for preparing the gelatin was based on the procedure described by Miyazaki, Ishitani, Takahashi, Shimoyama, Itoh & Attwood (2011), while the used assay volume (125 mL) was based on existing commercial gelatins forms. The used extract amount (and corresponding microspheres) was defined considering the DPPH scavenging activity EC_{50} of the free extract (EC_{50} = 86.17 μg/mL). Therefore, the gelatin was prepared at a concentration of 1% (1.25 g of *k*-carrageenan per 125 mL of distilled water) by heating up to 90 ^{o}C until complete dissolution. The following samples have been prepared: (i) two samples without adding the extract (control samples); (ii) two samples with free extract (considering the EC_{50}) and (iii) two samples with lyophilized microspheres (corresponding to the same amount of free extract). The free extracts and the lyophilized microspheres were added to the gelatin at 90 ^{o}C. The final products were frozen and lyophilized, for further evaluation of DPPH scavenging activity and reducing power, as previously described. An OM analysis was also performed to assess the integrity of the microspheres after gelatin preparation and lyophilisation.

Statistical analysis

In the phenolic compounds analysis and antioxidant activity evaluation, three samples of each plant material were used, while for the incorporation assays, two samples were prepared. All the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD), being analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 22.0 (IBM Corp., Armonk, NY, USA) program.

5.1.3. Results and discussion

Phenolic compounds in F. vesca hydromethanolic and aqueous extracts

Thirty individual phenolic compounds were detected and tentatively identified in the hydromethanolic and aqueous extracts prepared from commercial and wild samples of *F. vesca* vegetative parts (**Table 43**): twelve gallic/ellagic acid/HHDP derivatives, nine flavonols (*i.e.* quercetin and kampferol derivatives), eight flavan-3-ols (*i.e.*, catechins and proanthocyanidins) and one hydroxycinnamoyl derivative (*p*-coumaric acid derivative). The phenolic profiles of commercial and wild samples are very similar in terms of compound families, but with differences in individual compounds. Peaks 1, 3, 5, 8, 15, 20, 21, 24, 28 and 29 are common in both samples. An exemplificative phenolic profile of the infusion extract prepared from wild *F. vesca* is shown in **Figure 22**.

Ellagic and gallic acid derivatives

Ellagic acid derivatives represent the largest group of compounds found in the hydromethanolic extracts of commercial and wild samples of *F. vesca* vegetative parts. The total content of these compounds was higher than the one observed in the plant roots (Dias et al., 2015a), which confirms their differential accumulation in certain tissues (Clifford & Scalbert 2000).

Peak 28 was identified as ellagic acid according to its retention, mass and UV characteristics by comparison with a commercial standard. The rest of compounds of this group were tentatively identified based on their mass spectrum and comparison with data reported in the literature. Peaks 22 ($[M-H]^-$ at *m/z* 447) and 30 ($[M-H]^-$ at *m/z* 461) showed UV spectrum similar to ellagic acid and major MS^2 fragment ions at *m/z* 301 (ellagic acid) and 315, respectively, from the loss of 146 mu (deoxyhexosyl moiety); in the case of compound 30 a second fragment ion was observed at *m/z* 301, pointing to the further loss of a methyl group. These characteristics allowed their tentative identification as ellagic acid deoxyhexose and methyl ellagic acid deoxyhexose. Compounds with similar mass characteristics have been reported in fruits (Bubba et al., 2012; Gasperotti et al., 2013; Sun et al., 2014) and roots (Dias et al., 2015) of *F. vesca*, as well as in fruits of other *Fragaria* species (peak 22; Seeram, Lee, Scheuller & Heber, 2006; Aaby, Ekeberg & Skrede, 2007; Simirgiotis & Schmeda-Hirschmann, 2010).

The rest of the compounds of this group corresponded to hydrolysable tannins. Peaks 1 and 3 showed the same $[M-H]^-$ ion at *m/z* 783 and were identified as bis-HHDP-glucose isomers. The daughter ions at *m/z* 481 and 301 are commonly observed in the fragmentation pattern of ellagitannins and come respectively from the loss of a hexahydroxydiphenoyl unit (HHDP) followed by proton transfer, and the internal rearrangement of the HHDP itself

(Gasperotti et al., 2013). Similar compounds were previously reported in fruits of *Fragaria vesca* (Sun et al., 2014) and other *Fragaria* species (Seeram et al., 2006; Aaby et al., 2007; Simirgiotis and Schmeda-Hirschmann, 2010; Gasperotti et al., 2013), being usually associated to pedunculagin.

Peak 11 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 933 yielding main fragment ions at *m/z* 915, 631, 451 and 301, consistent with those described for castalagin/vescalagin isomers previously reported in roots (Dias et al., 2015a) and fruits (Bubba et al., 2012; Gasperotti et al., 2013) of *F. vesca*, as also in the leaves of *F. chiloensis* (Simirgiotis & Schmeda-Hirschmann, 2010). Peak 12 had a pseudomolecular ion $[M-H]^-$ at *m/z* 635 and MS^2 fragments ions at *m/z* 465 (loss of gallic acid, 170 mu), *m/z* 313 (further loss of a galloyl residue, 152 mu) and *m/z* 169 (gallic acid); based on this fragmentation pattern the compound was tentatively identified as trigalloylglucose, previously found in fruits of *F. vesca* by Sun et al. (2014).

Mass characteristics of peak 15 ($[M-H]^-$ at *m/z* 935 yielding fragments at *m/z* 633 and *m/z* 301) coincided with a galloyl-bis-HHDP-glucose isomer, previously reported in the roots (Dias et al., 2015a) and fruits of *F. vesca* (Bubba et al., 2012; Gasperotti et al., 2013; Sun et al., 2014) and associated to galloylpedunculagin or casuarictin/potentillin, one of the monomers frequently found as constituents of the oligomeric ellagitannins (Gasperotti et al., 2013). Peaks 16, 17 and 21 were assigned as Sanguiin h10 isomers, presenting a pseudomolecular ion $[M-H]^-$ at *m/z* 1567 and a characteristic fragmentation pattern at *m/z* 935, 633 and 301, which is in agreement with the identification made by Bubba et al. (2012), Gasperotti et al. (2013) and Dias et al. (2015a) in the fruits and roots of *F. vesca*. Peak 21 was the major compound found in both samples.

Peak 19, only observed in the commercial sample, showed a pseudomolecular ion $[M-H]^-$ at *m/z* 1235, with a subsequent loss of two HHDP units $[M-H-302-302]^-$ giving rise to fragments at *m/z* 933 and *m/z* 631, and then the loss of a glucose-galloyl unit $[M-H-330]^-$ yielding the fragment at *m/z* 301. A compound with similar characteristics was reported in strawberry fruits (*Fragaria x ananassa* Duch.) (Hanhineva et al. 2008; Aaby, Mazur, Nes & Skrede, 2012; Gasperotti et al., 2013) and tentatively associated di-HHDP-glucose-galloyl-ellagic acid, also designed as dauvriicin M1, a hydrolysable tannin previously identified in the roots *Rosa davurica* (Yoshida, Jin & Okuda, 1989).

Table 43. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectruml data, tentative identification and phenolic compounds quantification (mg/g) in the hydromethanolic and aqueous extracts prepared from commercial *F. vesca* vegetative parts

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Commercial			Wild		
						Hydromethanolic	Infusion	Decoction	Hydromethanolic	Infusion	Decoction
1	4.9	258	783	481(8),301(23)	Bis-HHDP-glucose	1.72 ± 0.22	0.77 ± 0.03	1.57 ± 0.23	1.03 ± 0.18	1.72 ± 0.12	0.79 ± 0.21
2	5.6	278	451	289(100)	(Epi)catechin hexoside	-	-	-	1.90 ± 0.02	4.51 ± 0.09	2.02 ± 0.18
3	5.8	260	783	481(10),301(38)	Bis-HHDP-glucose	1.41 ± 0.18	0.47 ± 0.10	0.91 ± 0.17	0.83 ± 0.01	0.63 ± 0.06	0.79 ± 0.09
4	7.0	278	865	713(11),695(10),577(11),575(13),289(10),287(19)	B-type (epi)catechin trimer	1.72 ± 0.14	4.05 ± 0.18	6.38 ± 0.24	-	-	-
5	7.3	280	577	451(23), 425(54),407(93), 289(58), 287(10)	Procyanidin dimer	5.86 ± 0.29	5.01 ± 0.07	3.38 ± 0.08	3.75 ± 0.05	8.47 ± 0.29	5.75 ± 0.08
6	7.1	280	865	713(8),695(17),577(18),575(16),289(5),287(10)	B-type (epi)catechin trimer	-	-	-	2.26 ± 0.09	4.82 ± 0.16	2.85 ± 0.23
7	7.7	356	639	463(69),301(59)	Quercetin hexose glucuronide	-	-	-	2.27 ± 0.05	4.04 ± 0.08	3.35 ± 0.05
8	8.1	280	289	245(80), 203(61), 137(37)	(+)-Catechin	2.01 ± 0.25	2.21 ± 0.22	1.80 ± 0.05	11.76 ± 0.19	21.65 ± 0.01	15.39 ± 0.08
9	9.7	278	561	435(27),407(30),289(80)	B-type (epi)afzelechin-(epi)catechin	-	-	-	2.64 ± 0.00	5.53 ± 0.04	3.58 ± 0.56
10	10.2	280	577	451(21), 425(43), 407(100), 289(72), 287(9)	Procyanidin dimer	-	-	-	3.04 ± 0.05	2.68 ± 0.21	2.42 ± 0.09
11	10.7	276	933	915(2),631(7),451(14)301(4)	Castalagin/Vescalagin	0.34 ± 0.02	-	-	-	-	-
12	11.3	264	635	465(100),313(18),295(2),169 (14)	Trigalloylglucose	0.10 ± 0.03	-	-	-	-	-
13	13.5	288	325	163(12),119(100),113(2)	*p*-Coumaroyl hexose	0.39 ± 0.02	0.36 ± 0.01	0.26 ± 0.01	-	-	-
14	14.7	278	561	435(28),407(37),289(80)	B-type (epi)afzelechin-(epi)catechin	-	-	-	2.10 ± 0.06	3.75 ± 0.29	3.84 ± 0.92
15	15.1	268	935	633(25),301(21)	Galloyl-bis-HHDP-glucose	2.43 ± 0.00	-	-	0.94 ± 0.03	-	-
16	15.8	268	1567	935(100),783(39),633(77),613(2),301(19)	Sanguiin h10 isomer	1.75 ± 0.04	-	-	-	-	-
17	16.8	268	1567	935(100),783(87),633(94),613(2),301(47)	Sanguiin h10 isomer	4.65 ± 0.10	1.38 ± 0.12	-	-	-	-
18	17.0	352	623	301(100)	Quercetin deoxyhexose glucuronide	-	-	-	8.51 ± 0.11	15.21 ± 0.08	13.57 ± 0.01
19	17.1	254/sh370	1235	933(13),631(6),301(6)	di-HHDP-glucose-galloyl-ellagic acid	2.57 ± 0.06	-	-	-	-	-
20	17.6	364	609	301(100)	Quercetin 3-*O*-rutinoside	4.27 ± 0.08	6.13 ± 0.06	5.67 ± 0.04	3.37 ± 0.03	5.11 ± 0.12	4.23 ± 0.02
21	18.6	264	1567	1265(7),1235(7),1085(39),935(100),783(27),633(6),613(2),301(16)	Sanguiin h10 isomer	17.87 ± 0.19	8.99 ± 0.30	8.49 ± 0.24	63.90 ± 0.89	7.40 ± 0.11	3.51 ± 0.05
22	19.7	250/sh370	447	301(100)	Ellagic acid deoxyhexose	0.91 ± 0.09	-	-	0.25 ± 0.07	-	-
23	19.8	346	607	285(100)	Kaempferol deoxyhexose	-	-	-	6.61 ± 0.12	11.96 ± 0.07	9.21 ± 0.05

					glucuronide						
24	20.6	358	477	301(100)	Quercetin *O*-glucuronide	5.07 ± 0.04	6.23 ± 0.16	6.23 ± 0.04	12.74 ± 0.11	22.10 ± 0.32	16.75 ± 1.20
25	20.4	354	637	315(95),300(26)	Methylquercetin deoxyhexose glucuronide	-	-	-	6.14 ± 0.40	10.43 ± 0.23	7.95 ± 0.11
26	21.1	356	463	301(100)	Quercetin 3-*O*-glucoside	-	-	-	0.59 ± 0.00	1.41 ± 0.06	0.53 ± 0.01
27	21.2	348	593	285(100)	Kaempferol 3-*O*-rutinoside	3.22 ± 0.01	4.97 ± 0.00	5.56 ± 0.10	0.69 ± 0.08	-	0.15 ± 0.04
28	21.7	252/sh370	301	284(16),256(11),229(18), 185(11)	Ellagic acid	1.66 ± 0.06	2.37 ± 0.02	4.08 ± 0.33	1.18 ± 0.02	1.77 ± 0.02	1.40 ± 0.02
29	24.8	350	461	285(100)	Kaempferol *O*-glucuronide	0.79 ± 0.01	1.05 ± 0.01	1.05 ± 0.01	-	-	-
30	26.1	248/sh372	461	315(89),301(38)	Methyl ellagic acid deoxyhexose	-	-	-	1.85 ± 0.01	1.47 ± 0.00	0.54 ± 0.02
					Total Ellagic Acid derivatives	35.31 ± 0.84^{a}	13.98 ± 0.29^{c}	15.06 ± 0.48^{b}	69.49 ± 1.18^{a}	11.22 ± 0.06^{b}	5.78 ± 0.27^{c}
					Total Flavonols	13.35 ± 0.01^{b}	18.38 ± 0.11^{a}	18.51 ± 0.11^{a}	41.42 ± 0.03^{c}	72.02 ± 0.40^{a}	56.98 ± 1.11^{b}
					Total Phenolic Acid derivatives	0.39 ± 0.06^{a}	0.36 ± 0.01^{b}	0.26 ± 0.01^{c}	-	-	-
					Total Flavan 3-ols	9.59 ± 0.09^{b}	11.27 ± 0.03^{a}	11.56 ± 0.22^{a}	27.46 ± 0.01^{c}	51.41 ± 0.44^{a}	35.83 ± 0.52^{b}
					Total Phenolic Compounds	58.73 ± 0.83^{a}	43.99 ± 0.37^{c}	45.38 ± 0.80^{b}	138.37 ± 1.20^{a}	134.65 ± 0.09^{b}	98.59 ± 0.85^{c}

For the total compounds, in each row and for each sample (commercial or wild), different letters mean significant statistical differences between samples ($p<0.05$), where “a” and “c” correspond to the highest and lowest values, respectively.

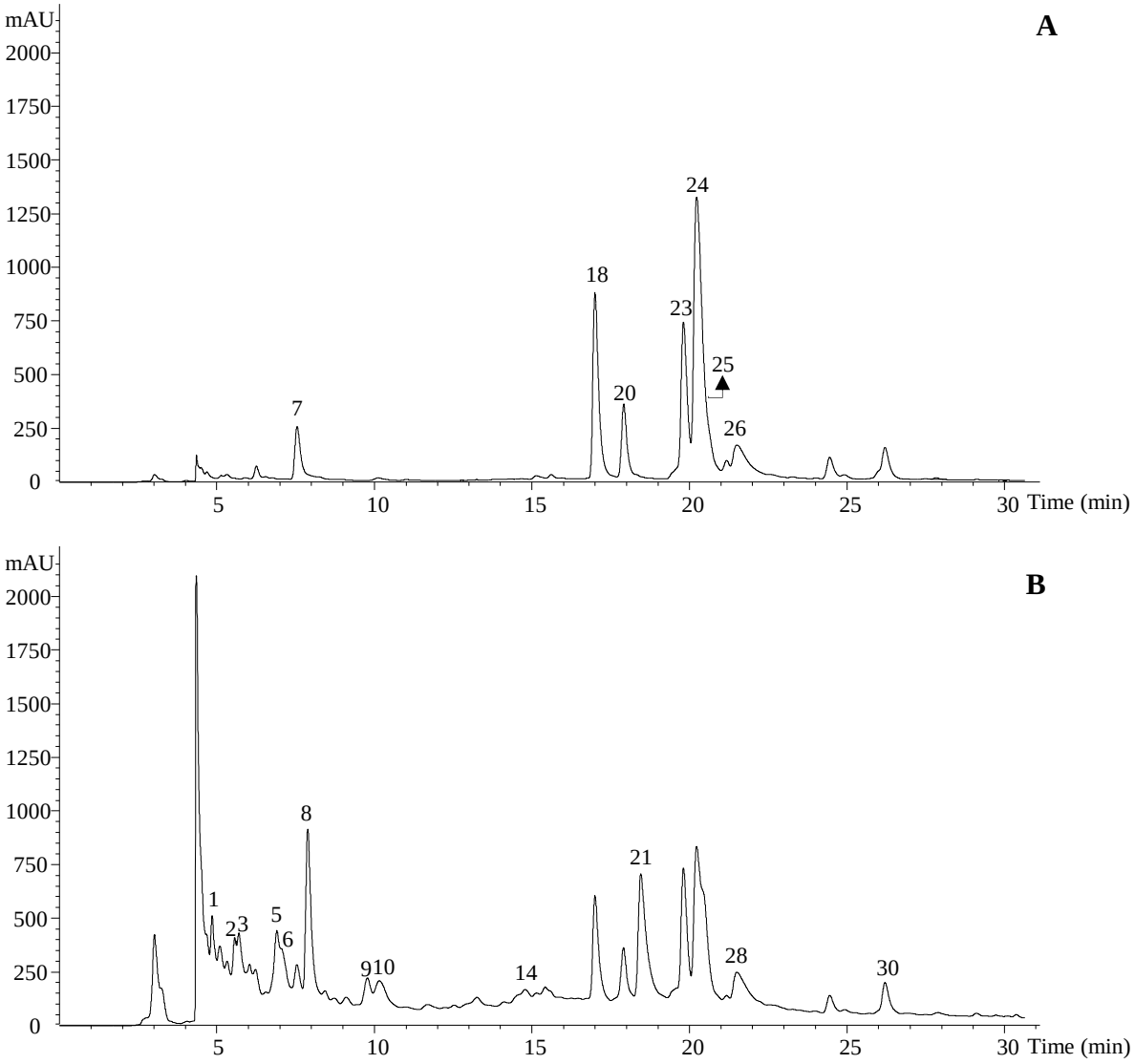

Figure 22. HPLC phenolic profile of the infusion extract obtained from wild *F. vesca* vegetative parts, obtained at 370 nm (A) and 280 nm (B).

Flavonols

Flavonols represent the second largest group of phenolic compounds found in the hydromethanolic extracts, but the largest group in the aqueous extracts obtained from both commercial and wild samples. Quercetin (peaks 7, 18, 20, 24 and 25), kampferol (peaks 23, 27 and 29) and methylquercetin (peak 26) derivatives were the main flavonols found. Peaks 7, 18, 23, 25 and 26 were only found in the wild sample, while peak 27 was only detected in the commercial one.

Peaks 20 (quercetin 3-*O*-rutinoside), 26 (quercetin 3-*O*-glucoside) and 27 (kaempferol 3-*O*-rutinoside) were positively identified by comparison of their retention, mass and UV-vis characteristics with commercial standards. The presence of quercetin 3-*O*-glucoside was

described in roots (Dias et al., 2015a) and fruits (Sun et al., 2014) of *F. vesca*. A peak with the same pesudomolecular ion as peak 27 ($[M-H]^-$ at *m/z* 593) was also reported in *F. vesca* fruits (Bubba et al., 2012; Sun et al., 2014) and in other *Fragaria* species (Seeram et al., 2006; Simirgiotis & Schmeda-Hirschmann, 2010; Aaby et al., 2012), but identified as kaempferol-coumaroylhexoside, identity that was discarded in our case once the compound was compared with a standard of kaempferol 3-*O*-rutinoside and lacked in its UV spectrum the characteristic shoulder of the *p*-coumaroyl substituent expected around 310 nm. As far as we know, the presence of kaempferol 3-*O*-rutinoside has not been cited in *F. vesca.*

Mass characteristics of peak 24 ($[M-H]^-$ at *m/z* 477 yielding a unique MS^2 fragment at *m/z* 301) were coherent with quercetin *O*-glucuronide, compound that was previously identified in the fruits of *F. vesca* (Bubba et al., 2012; Sun et al., 2014) and other *Fragaria* species (Simirgiotis & Schmeda-Hirschmann, 2010; Aaby et al., 2012). Similar behaviour was found for compound 29 ($[M-H]^-$ at *m/z* 461 yielding an MS^2 fragment at *m/z* 285 from the loss of a glucuronyl residue) that was thus identified as kaempferol *O*-glucuronide, already described in the fruits of *F. vesca* (Sun et al., 2014) and other *Fragaria* species (Seeram et al., 2006; Simirgiotis & Schmeda-Hirschmann, 2010; Aaby et al., 2012).

Peak 7 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 639 with fragments at *m/z* 463 (loss of a glucuronyl group) and *m/z* 301 (further loss of an hexosyl residue), being tentatively identified as quercetin hexose glucuronide. A similar compound was reported in strawberry flowers by Hanhineva et al. (2008). Peak 18 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 623, releasing MS^2 fragment ions at *m/z* 301 ($[M-H-322]^-$), which might correspond to the joint loss of deoxyhexosyl (-146 mu) and glucuronyl (-176 mu) groups, so that the compound was tentatively assigned as quercetin deoxyhexose glucuronide. Similar loss of 322 mu (176+146 mu) was observed for peaks 23 ($[M-H]^-$ at *m/z* 607 yielding an MS^2 fragment at *m/z* 285) and 25 ($[M-H]^-$ at *m/z* 637 releasing a major MS^2 fragment ion at *m/z* 315 and a minor one at *m/z* 300, further loss of a methyl group), which allowed their tentative identification as kaempferol deoxyhexose glucuronide and methylquercetin deoxyhexose glucuronide, respectively. As far as we know, these latter three compounds have been previously reported in *F. vesca* or other *Fragaria* species (Simirgiotis & Schmeda-Hirschmann, 2010; Aaby et al., 2012).

Flavan-3-ols

Peak 8 was positively identified as (+)-catechin according to its retention time, mass and UV-vis characteristics by comparison with a commercial standard. Peak 2 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 451 releasing an MS^2 fragment at *m/z* 289 ($[M-H-162]^-$, loss of a hexosyl moiety), corresponding to an (epi)catechin monomer, being tentatively identified as (epi)catechin hexoside. The earlier elution of this compound comparatively to

peak 8 (parent aglycone) is in agreement to its higher polarity (presence of a sugar). A compound with similar characteristics was detected in *F. vesca* roots (Dias et al., 2015a) and fruits (Bubba et al., 2012) and given the same tentative identity.

Peaks 4, 5, 6, 9, 10 and 14 were identified as proanthocyanidins (PAC) based on their pseudomolecular analysis and MS^2 fragmentation patterns. The analysis of the produced fragments provides information about the type of elementary units and also about their relative position in the PAC oligomer; however, mass spectrometry does not provide the enough information to establish the position between flavonol units (i.e. C4-C8 or C4-C6) and does not differentiate between isomeric catechins. Peaks 5 and 10 were identified as procyanidin dimers, presenting the same pseudomolecular ion $[M-H]^-$ at *m/z* 577 and MS^2 fragmentation patterns coherent with B-type (epi)catechin dimers. Characteristic product ions were observed at *m/z* 451 (-126 mu), 425 (-152 mu) and 407 (-152 to 18 mu), attributed to the HRF (heterocyclic ring fissions), RDA (retro-Diels-Alder) and further loss of water from an (epi)catechin unit, and at *m/z* 289 and 287, that could be associated to the fragments corresponding to the lower and upper (epi)catechin unit, respectively. Peaks 4 and 6 were identified as B-type (epi)catechin trimers with pseudomolecular ions $[M-H]^-$ at *m/z* 865, producing characteristic MS^2 fragmentation ions at *m/z* 289 and 287. Additional fragments were observed at *m/z* 713, 695, 577 and 575, corresponding to the alternative HRF, RDA and interflavan bonds cleavages. Peaks 9 and 14 were tentatively assigned as B-type (epi)afzelechin-(epi)catechin, presenting a pseudomolecular ion $[M-H]^-$ at *m/z* 561 and characteristic fragment ions at *m/z* 435, 407 and 289.

Similar proanthocyanidins to the mentioned above have been previously reported in commercial and wild samples of *F. vesca* roots (Dias et al., 2015a) and fruits (Simirgiotis & Schmeda-Hirschmann, 2010; Bubba et al., 2012; Sun et al., 2014), as well as in other *Fragaria* species (Määttä-Riihinen et al., 2004; Seeram et al., 2006; Hanhineva et al., 2008; Simirgiotis & Schmeda-Hirschmann, 2010; Aaby et al., 2007, 2012). As observed for total flavonols, the aqueous extracts showed higher quantities of total flavan 3-ols than the hydromethanolic extracts.

Phenolic acids derivatives

Finally, peak 13, only detected in the commercial sample, was tentatively identified as *p*-coumaric hexose based on its pseudomolecular ion $[M-H]^-$ at *m/z* 325 releasing a daughter ion at *m/z* 163 ($[\text{coumaric acid-H}]^-$) from the loss of a hexosyl moiety ($[M-H-162]^-$). A compound with similar characteristics was reported to occur in different strawberry (*Fragaria x ananassa* Duch.) varieties (Määttä-Riihinen et al., 2004; Seeram et al., 2006; Aaby et al., 2007, 2012; Sun et al., 2014).

Antioxidant activity of F. vesca hydromethanolic and aqueous extracts

The aqueous extracts of both samples (commercial and wild) gave higher antioxidant activity than the corresponding hydromethanolic extracts (**Table 44**). This was observed in all the assays: DPPH scavenging activity, reducing power, β-carotene bleaching inhibition and TBARS formation inhibition. Nevertheless, in commercial samples the aqueous extract obtained by decoction was the most active, while for the wild samples it was the extract obtained by infusion that gave the highest activity. Therefore, the antioxidant activity seems to be more related with the flavonoids content (flavonols and flavan-3-ols) than with ellagic acid levels, since aqueous extracts gave higher amounts of flavonoids than the hydromethanolic extracts (in both commercial and wild samples) (**Table 43**).

It should be noticed that all the extracts prepared from wild samples showed, in all the assays, higher antioxidant activity than the correspondent extracts from commercial vegetative parts (**Table 43**). This is certainly related to the higher content of the wild samples in phenolic compounds that are secondary metabolites with increased production under adverse and non-controlled conditions. In a study with *F. vesca* roots, the authors observed this same behaviour (Dias et al., 2015a).

The antioxidant activity of other Fragaria species and parts was previously reported namely, DPPH scavenging activity of *F. chiloensis ssp. chiloensis f. chiloensis* leaves and roots (Simirgiotis & Schmeda-Hirschmann, 2010), and *F. vesca* leaves (Žugic et al., 2014).

Table 44. Antioxidant activity of the hydromethanolic and aqueous extracts obtained from commercial and wild *F. vesca* vegetative parts.

	Commercial			Wild			Trolox
EC_{50} values (µg/mL)	**Hydromethanolic**	**Infusion**	**Decoction**	**Hydromethanolic**	**Infusion**	**Decoction**	
DPPH scavenging activity	139.33 ± 2.61[a]	121.94 ± 6.40[b]	118.89 ± 1.13[c]	123.67 ± 7.92[a]	86.17 ± 2.42[c]	109.10 ± 1.28[b]	43.03 ± 1.71
Reducing power	324.49 ± 2.20[a]	91.88 ± 1.33[b]	88.20 ± 0.50[c]	81.40 ± 2.43[a]	62.36 ± 1.43[c]	77.28 ± 3.13[b]	29.62 ± 3.15
β-carotene bleaching inhibition	388.90 ± 15.06[a]	76.41 ± 0.66[b]	69.98 ± 2.65[c]	56.71 ± 0.66[a]	12.34 ± 1.62[c]	13.40 ± 1.81[b]	2.63 ± 0.14
TBARS inhibition	24.36 ± 0.68[a]	23.07 ± 0.40[b]	17.52 ± 0.31[c]	12.63 ± 0.77[a]	3.12 ± 0.17[c]	5.03 ± 0.06[b]	3.73 ± 1.9

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. For the total compounds, in each row and for each sample (commercial or wild), different letters mean significant statistical differences between samples ($p<0.05$), where “a”and “c” correspond to the highest and lowest values, respectively.

The extract of *F. vesca* vegetative parts showing the highest antioxidant activity (infusion from wild samples) was used in the development of a nutraceutical formulation for further application in *k*-carrageenan gelatin. This is an attractive approach since aqueous extracts are more suitable for food applications than the hydromethanolic ones.

Alginate microspheres with F. vesca infusion extract

Microspheres production, morphology and encapsulation efficiency

The atomization/coagulation technique, spray-based process, was used to prepare alginate-based microspheres containing infusion extracts of wild *F. vesca* vegetative parts. Immediately after the atomization and the coagulation steps, the produced microspheres were analysed by OM (**Figure 23 A** and **B**). In the first stage, atomization, the microspheres presented a high degree of teardrop-shaped due to the passage through the equipment nozzle. After 4 hours of coagulation the microspheres' shape becomes spherical. In both stages, the microspheres were perfectly individualized (no agglomerates were detected). Their final estimated size (using a magnification of 400X) ranged between 39 and 202 μm. With the incorporation of the infusion extract the microspheres presented a light brown colour, characteristic of the used extract, which indicates its incorporation and a good distribution inside the microspheres. The encapsulation efficiency (EE) determination, based on quercetin-*O*-glucuronide, was done by HPLC by analysing and conducted to a value close to 97%. A SEM analysis was also performed on the final lyophilized microspheres. As it can be observed in the shown micrographs (**Figure 23 E**), the microspheres have spherical shape and a rough surface. The observed round cavities are due the proximal presence of other particles during the drying process. It was also observed (data not shown) that microspheres containing no extract have the tendency to collapse giving rise to particles with a disc-like morphology. This type of morphology was not noticed for microspheres incorporating the extract.

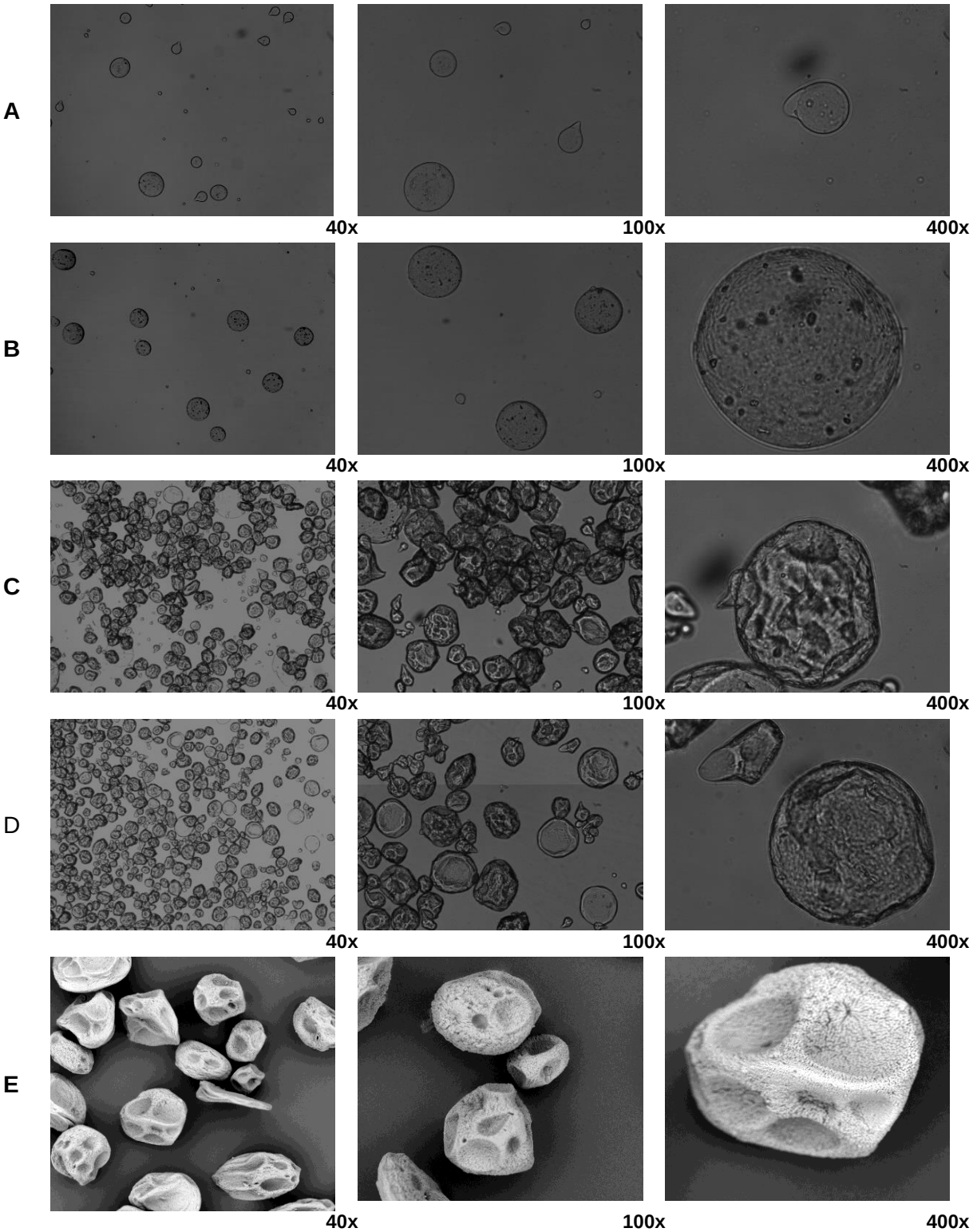

Figure 23. OM analysis with magnifications of 40, 100 and 400× of the microspheres immediately after atomization (A), after 4 hours coagulation period under stirring at 400 rpm (B), lyophilized microspheres (C), after 48 hours rehydration (D); and SEM analysis with magnification of 550, 1000 and 2000x (E).

Microspheres rehydration after lyophilisation

To test the rehydration capacity and, consequently, the initial morphology recovery, the lyophilized microspheres were rehydrated with distilled water for a period of 48 hours. An OM analysis was made for dried and rehydrated forms using the magnifications of 40, 100 and 400X. The rehydrated microspheres practically acquired the same initial shape and size (**Figure 23 C** and **D**), proving to have a good rehydration capacity. The water recovery after 48 hours of rehydration was close to 100%.

Fourier transform infrared spectroscopy (FTIR)

The FTIR spectrum of pure alginate, pure infusion extract and microspheres incorporating the extract, are shown in **Figure 24**. The microsphere's spectrum, as expected, is dominated by the presence of alginate (dotted orange lines). The ratio extract/alginate was 100/800, which explains the alginate preponderance. Nevertheless a noticeable contribution from both carbonyl (C=O) and hydroxyl (OH) groups of the extract (dotted green lines) was observed. Also a widening of the OH and C=O bands can be observed. These facts represent an evidence of effective extract encapsulation.

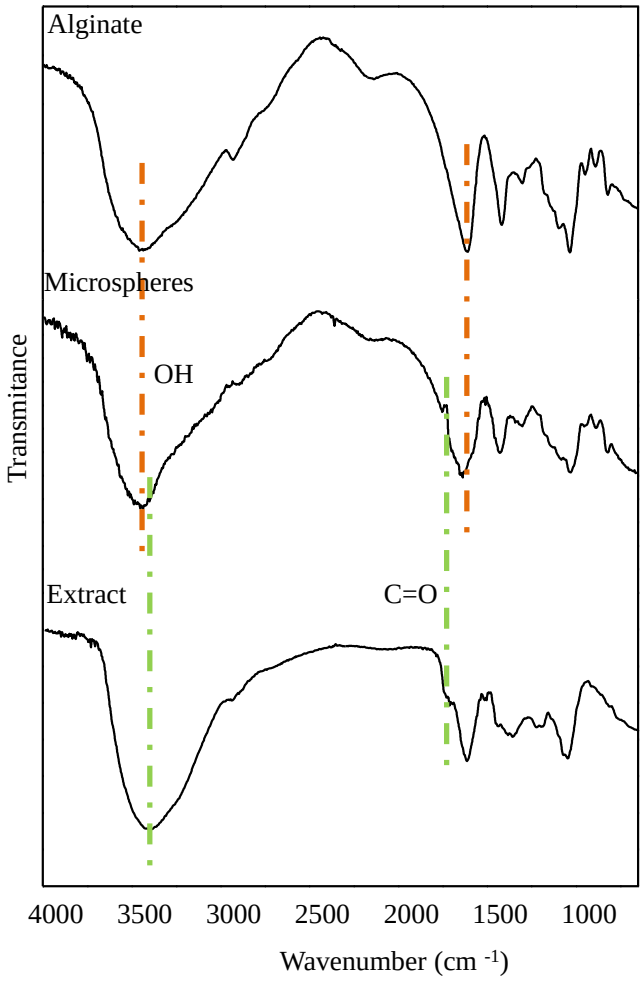

Figure 24. FTIR spectrum of pure alginate, pure infusion extract and microspheres enriched with the infusion extract

Application in k-carrageenan gelatin

Figure 25 A and **B** show, respectively, the morphology of the enriched microspheres immediately after incorporation in the *k*-carrageenan gelatin and after subsequent lyophilisation. It can be observed that the temperature used to prepare the gelatin solution (90 ºC) did not affect the microsphere's integrity that shown a perfect round shape as a result of a prompt rehydration. After lyophilisation the spherical structure was maintained. Also it is clearly the presence of dark black dots inside the microspheres representing the encapsulated extract, showing the effective protective effect of the alginate matrix.

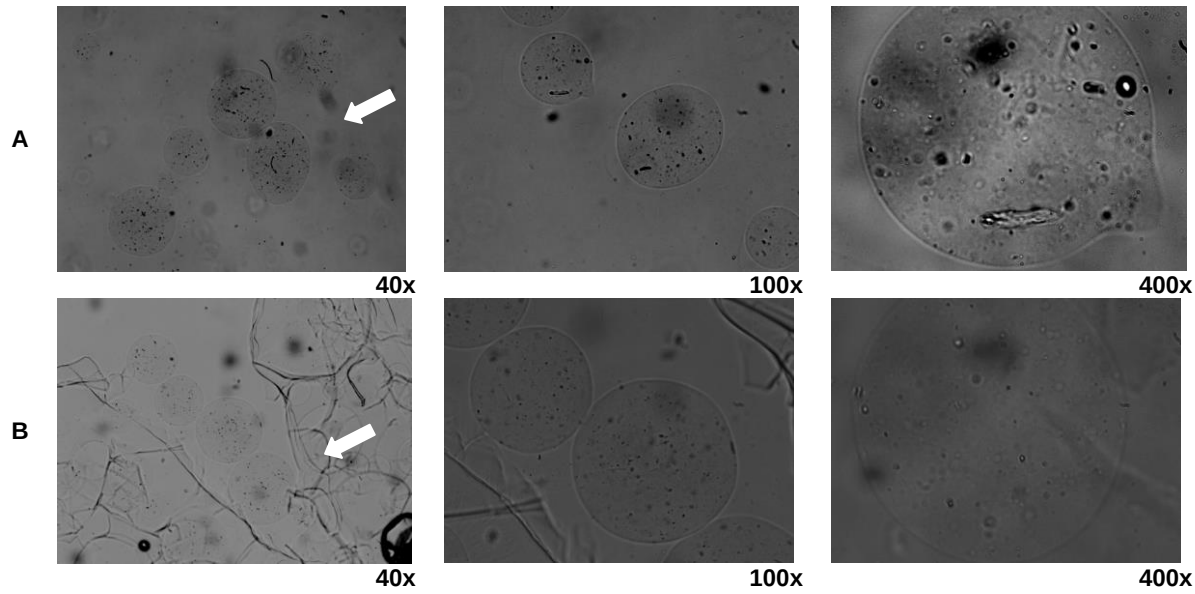

Figure 25. OM analysis with magnification of 40, 100 and 400× of k-carrageenan with microencapsulated infusion extract before (**A**) and after (**B**) lyophilisation

Regarding the antioxidant activity of the final product, evaluated by DPPH scavenging activity and reducing power, as expected, only *k*-carrageenan gelatin enriched with the free (non-encapsulated) infusion extract showed antioxidant activity (EC_{50} DPPH scavenging activity = 2.74±0.11 mg/mL; EC_{50} reducing power = 1.23±0.12 mg/mL). Nevertheless, a loss of antioxidant activity, relatively to the extract in its free form, was noticed possibly due to the high temperatures needed to prepare the gelatin, which lead to extract degradation. Neither the control nor the gelatin with microencapsulated extracts showed antioxidant activity. The first result (control) was predictable since no antioxidant additives were present. In the second case (microencapsulated extract) the result is justified by an efficient protection of the alginate microspheres. In fact, the extract was effectively protected inside the alginate microspheres by the help of a surrounding viscous medium (gelatin) that hinders its easy diffusion. It is therefore expected that this kind of nutraceutical formulation (gelatin enriched with alginate-based microencapsulated extracts) works well for liberation at pH=7.4 (intestinal preferable absorption) since at this pH the alginate microspheres lose this integrity (disruption of the ionic polymeric network) and liberate the encapsulated extracts.

Overall, wild samples of *F. vesca* vegetative parts showed higher contents in phenolic compounds and higher antioxidant activity than the commercial ones. Aqueous preparations were more active than hydromethanolic extracts due to the higher amounts of flavonols and flavan-3-ols. The microencapsulation technique of atomization/coagulation was effectively applied to produce microspheres enriched with the most antioxidant extract, the infusion from wild *F. vesca* (encapsulation efficiency close to 95%). The incorporation of the microspheres

into a gelatin food matrix proved that this system preserves the antioxidant properties of the extract as compared with the free form. This is an innovative study on the development of nutraceuticals based on *F. vesca* extracts. Further studies will be required to establish a controlled release of the bioactive extract within the organism, using an *in vitro* gastrointestinal model.

Competing interests

The authors declare no competing financial interest.

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6.

Considerações finais e perspetivas futuras

Neste capítulo final descrevem-se as conclusões obtidas em cada um dos capítulos envolvendo trabalho experimental, culminando com uma conclusão global sobre o trabalho desenvolvido onde se faz uma anáise sobre as potencialidades dos resultados e a sua aplicação na indústria alimentar. Numa perspetiva de continuidade deste trabalho, apresentam-se também sugestões de trabalho futuro.

6.1. Conclusão geral

Este trabalho teve como objetivo a aplicação de duas ferramentas na área dos bioativos: a técnica de cultura de células e tecidos como um meio de produção sustentável de compostos bioativos a larga escala e a microencapsulação como uma metodologia de proteção dos bioativos viabilizando a sua aplicação em matrizes alimentares. Este trabalho abordou, portanto, duas grandes problemáticas associadas á utilização dos bioativos, a sua obtenção a partir de matrizes naturais sem comprometer a biodiversidade e respetivos habitats, e a manutenção das suas propriedades bioativas ao longo do processamento, armazenamento e ingestão dos alimentos. Assim para colocar em prática as duas técnicas foram selecionadas plantas utilizadas na medicina tradicional reconhecidas pelos seus efeitos benéficos para a saúde humana. Após a caraterização química das plantas eleitas, e uma vez realizado o *screening* das suas propriedades bioativas, procedeu-se à aplicação das técnicas de cultura de células e tecidos e microencapsulação à espécie vegetal que se mostrou mais promissora: *Fragaria vesca* L. Adicionalmente, foi objetivo também de este trabalho percorrer toda a cadeia produtiva de um alimento funcional, nomeadamente desde a obtenção do extrato até à sua incorporação e validação numa matriz alimentar. Todos os objetivos delineados para esta tese foram alcançados, apresentando-se resultados promissores para futuros projetos na área alimentar, mas também extensíveis a outras áreas industriais.

6.2. Conclusões parciais

6.2.1. Composição química e propriedades bioativas das espécies vegetais

O *screening* inicial, feito a várias plantas tradicionalmente consumidas no Nordeste Transmontano, na sua forma desidratada ou sob a forma de extratos hidrometanólicos e aquosos (infusão e decocção), revelou que todas as amostras apresentam elevado potencial para serem utilizadas como fonte de nutrientes e de compostos bioativos. Revelou também que algumas plantas apresentam, adicionalmente, potencial para serem utilizadas como citotóxicas para células tumorais (*Achillea millefolium* L. e *Laurus nobilis* L.), antifúngicas (*Laurus nobilis* L.) e antibacterianas (*Laurus nobilis* L. *e Fragaria vesca* L.). Destacam-se ainda os seguintes aspetos:

- As plantas silvestres, comparativamente às comerciais, revelaram um potencial superior como fontes de compostos nutracêuticos e bioativos, sendo esta observação válida para todas as espécies estudadas;
- Os extratos aquosos (infusão e decocção) mostraram resultados promissores ao nível da bioatividade e como fonte de compostos fenólicos;

- Os estudos de digestão *in vitro* aplicados à fração mineral revelaram que apenas uma pequena parte destas substâncias permanece bioacessível após ingestão.

6.2.2. Utilização da cultura *in vitro* para estimular a produção de bioativos

O estabelecimento da cultura *in vitro* de *Fragaria vesca* L. foi realizado para obtenção de partes vegetativas, posteriormente analisadas em termos das suas características nutricionais, químicas e bioativas. As plantas produzidas por esta técnica apresentaram várias vantagens quando comparadas com as correspondentes amostras silvestres, nomeadamente:

- Teor superior em proteínas, ácidos gordos polinsaturados, açúcares, ácidos orgânicos e tocoferóis;
- Para os extratos hidrometanólicos, atividade antioxidante superior;
- Identificação de outros compostos fenólicos que mostraram estar correlacionados com a atividade antioxidante.

6.2.3. Microencapsulação de bioativos e incorporação numa matriz alimentar

O desenvolvimento de uma nova formulação nutracêutica foi conseguida pelo uso de microesferas de alginato enriquecidas com o extrato obtido a partir da infusão das partes vegetativas de *Fragaria vesca* L. silvestre, posteriormente incorporadas numa gelatina. Dos resultados obtidos, destacam-se os seguintes pontos:

- Os extratos obtidos a partir da infusão, posteriormente selecionados para microencapsular, demonstraram ser aqueles com atividade antioxidante superior em todos os ensaios. Tal pode estar relacionado com a presença de flavonois e flavan-3-óis;
- A técnica de atomização/coagulação demonstrou ser eficaz para a encapsulação do extrato selecionado, tendo sido obtida uma eficiência de encapsulação de aproximadamente 95% (m/m);
- A integridade e capacidade de reidratação das microesferas foi mantida após preparação da gelatina *k*-carragenina (100 $^{\circ}$C);
- A gelatina com o extrato livre apresentou menor bioatividade, revelando que a temperatura requerida para a sua preparação, pode ter levado à degradação do extrato;
- A gelatina contendo o extrato microencapsulado não revelou qualquer atividade antioxidante, significando que este ficou protegido no interior das microesferas. É de esperar após ingestão da gelatina este seja libertado mantendo intacta a sua bioatividade.

6.3. Perspetivas futuras

As plantas aromáticas e medicinais apresentam propriedades nutricionais, químicas e bioativas que lhes conferem grande potencial de aplicação na indústria alimentar, assim como em outros setores industriais. Existem uma infinidade de espécies e variedades, para além das variações genéticas dentro das mesmas, pelo que o estudo deste tipo de matrizes naturais deve ser contínuo. A procura de novas fontes de compostos bioativos apresenta-se assim como o seguimento lógico deste trabalho.

As técnicas de cultura de células e tecidos vegetais, apesar de morosas, demonstram ser viáveis para a produção de compostos bioativos em larga escala sem comprometer as culturas silvestres e evitando a sobre-exploração dos solos. Assim, é importante otimizar a obtenção das culturas desejadas, nomeadamente utilizando novas formas de elicitação para obter compostos com elevado potencial bioativo. É fundamental ultrapassar a fase estacionária do crescimento das plantas para que haja um incremento da produção de compostos.

A aplicação de bioativos encapsulados em matrizes alimentares, de uma forma mais generalizada, representa também um tópico de interesse para estudos futuros. As aplicações a desenvolver podem ser variadas exigindo uma maior compreensão das interações entre o bioativo, o material encapsulante e a matriz alimentar. Adicionalmente, os estudos de libertação controlada e digestão *in vitro* são de extrema importância para a compreensão da bioacessibilidade dos compostos bioativos microencapsulados após ingestão.

Notas finais

Apesar das duas técnicas apresentadas nesta tese serem consideradas viáveis para o desenvolvimento de novos ingredientes para a indústria alimentar, devem realçar-se os seguintes aspetos:

i) A técnica de cultura de células e tecidos existe desde meados do século XX e, desde então, a sua área de aplicação tem sido, principalmente, o campo da fisiologia vegetal, nomeadamente para a compreensão de algumas vias biossintéticas. Adicionalmente tem sido aplicada de forma extensiva à indústria da floricultora e plantas para uma obtenção rápida e em quantidade de clones de espécies selecionadas. A sua aplicação para a produção de compostos bioativos tem vindo a ser explorada mais recentemente, existindo

atuamente no mercado produtos resultantes da cultura *in vitro* de plantas. No entanto, face a todas as vantagens da técnica, pode considerar-se que está ainda sub-explorada no campo da obtenção de compostos bioativos para aplicação na indústria alimentar.

ii) Atualmente, existem ainda lacunas na legislação Europeia no que respeita à autorização do uso de certos materiais encapsulantes e quanto à utilização dos microencapsulados em matrizes alimentares. Isto impede o investimento industrial, nomeadamente o do setor alimentar, para o desenvolvimento de novos produtos baseados na microencapsulação de bioativos. A consciencialização da importância e impacto destes produtos na promoção da saúde é uma etapa importante para a sua legislação.